α7 nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies

α7 nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies

Nick Petrus van Goethem

α7 nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies

ISBN: 978-90-9029038-6
Cover design by Ilona van Goethem
Cover image “Hippocampal Neurons” by Dr. Robert E. Clark, University of California, San Diego. Used with permission.

Production: CPI Koninklijke Wöhrmann B.V. te Zutphen

α7 nicotinic acetylcholine receptors and memory processes: mechanistic and behavioral studies

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de Rector Magnificus, Prof. dr. L.L.G. Soete, volgens het besluit van het College van Decanen, in het openbaar te verdedigen op donderdag 25 juni 2015 om 14:00 uur

door

Nick Petrus van Goethem
geboren op 16 januari 1984
te 's-Hertogenbosch

Promotor

Prof. Dr. H.W.M. Steinbusch

Copromotor

Dr. J. Prickaerts

Beoordelingscommissie

Prof. Dr. T. van Amelsfoort, voorzitter
Prof. Dr. M. de Baets
Dr. A. Blokland
Dr. E. Fedele, Genoa Italy
Prof. Dr. H. Mansvelder, VU Amsterdam

The studies described in this dissertation were partly supported by a Kootstra Talent Fellowship

Financial supports for the publication of this dissertation by “Alzheimer Nederland”, “Internationale Stichting Alzheimer Onderzoek (ISAO)” and the “School for Mental Health and Neuroscience, Maastricht University” are gratefully acknowledged.

“The existence of forgetting has never been proved:
We only know that some things don't come to mind
when we want them.”

Friedrich Nietzsche (1844-1900)

Contents

Chapter 1

General Introduction

What is cognition? Cognition is often described as a term which refers to the mental processes which are involved in knowledge gaining and comprehension. As such, cognition involves memory, attention, executive functions, perception, language and psychomotor functions (Nehlig, 2010). A complete range of cognitive abilities was set by Millan et al. in their 2012 review. Here cognition is defined as "a suite of interrelated conscious and unconscious mental activities, including pre-attentional sensory gating, attention, learning and memory, problem solving, planning, reasoning and judgment, understanding, knowing and representing, creativity, intuition and insight, spontaneous thought, introspection, as well as mental time travel, self-awareness and meta cognition, i.e. thinking and knowledge about cognition" (Froestl et al., 2012; Millan et al., 2012). Based on this, it is evident that everyday living relies heavily upon cognitive abilities.

Cognitive dysfunction and cognition enhancers

Cognitive dysfunction is a feature often encountered in a broad spectrum of neurological and psychiatric conditions. These cognitive deficits are commonly found in patients suffering from Alzheimer's disease (AD), schizophrenia, stroke, attention deficit hyperactivity disorder (ADHD) or aging (Froestl et al., 2012). Ameliorating these dysfunctions can dramatically improve the quality of life of patients. Hence, developing treatments, or 'cognition enhancers' (nootropics), is imperative in this respect.

'Cognition enhancers' is an overarching term used for drugs that treat cognitive dysfunction. These drugs are not a cure for the disease or disorder they were designed for, but can relief the patient to a great extent of suffering the cognitive symptoms of their condition (i.e. symptomatic treatment). Accordingly, over the last few decades certain drugs have been approved for the treatment of cognitive impairments related to specific neurological and psychiatric conditions and specific cognitive abilities including memory and attention.

Animal models and tests

A diverse range of animal models is being used to identify potential cognition enhancing drugs. In these animal models, cognitive deficits are often induced in order to investigate whether these deficits can be ameliorated experimentally. Such models can be based on pharmacological deficits, the naturally occurring aging process and/or introduction of transgenic constructs in rodents.

In pharmacological deficit models, specific drugs are administered to animals in order to induce cognitive deficits. The targets of these cognition-impairing drugs are hypothesis-based and are often directed to alter distinct neurotransmitter systems, with different disorders showing specific dysregulations or impairments.

After an appropriate animal model is chosen, the animals need to be tested in a paradigm which is specifically designed to measure the cognitive ability of interest. There are tests which are specifically designed to measure different cognitive abilities.

A widely used test is the object recognition task (ORT), which allows assessing learning and memory processes in rodents (Ennaceur and Delacour, 1988). The ORT is based on 'spontaneous novelty preference', i.e. the natural

predisposition of rodents to explore novel objects more than familiar objects. Different rodent species appear to perform at a similar level in this task and therefore, the ability to discriminate between familiar and novel objects may be a common ability among rodent species. The ORT allows assessing the effects of experimental interventions, such as lesioning of selected brain areas (Mumby et al., 2002), genetic manipulations (Vaucher et al., 2002), administration of compounds that impair recognition performance (Dodart et al., 1997; Norman et al., 2002), or of putative cognition enhancers (Ennaceur et al., 1989; Norman et al., 2002; Prickaerts et al., 2002a, 2002b) on learning and memory processes in rodents. Therefore, this test is very well suited to assess the effects of cognition enhancing drugs on learning and memory processes in different rodent models of psychiatric and neurological conditions. Hence, this test is often used for identifying memory enhancing drugs for the symptomatic treatment of for example AD and schizophrenic patients.

Neuronal nicotinic acetylcholine receptors

Nicotine has been shown to improve attention, learning and memory through interaction with neuronal nicotinic acetylcholine receptors (nAChRs) (Levin et al., 2006). nAChRs are distributed throughout the central nervous system (CNS) and are members of the super family of ligand-gated ion channels. To this family also belong the $GABA_A$, glycine and the serotonin type 3 ($5\text{-}HT_3$) receptors, all signature Cys-loop proteins (Cascio, 2004). These receptors are ionotropic receptors, and are directly linked to ion channels. As such, ligand-gated ion channels have the ligand-binding site and the ion channel located within the same protein structure, this accounts for ligand-binding and channel opening to occur simultaneous. Hence this macromolecule constitutes a complete pharmacological system (Hogg and Bertrand, 2007).

Each nAChR consists of five subunits, and in the CNS these subunits can either be of the α- or the β-subtype. To date, eight α- (α2 – α7, α9 and α10) and three β-subunits (β2 – β4) have been identified in the mammalian brain. These subunits can combine to from functional ion channels, which constitute the family of nAChR subtypes. Several subtypes of nAChRs are expressed in the mammalian brain, each of them displaying distinct physiological and pharmacological properties. Functional nAChRs are assembled from five subunits around an axis of pseudosymmetry and can be composed of identical subunits (homopentamers) or different subunits (heteropentamers) (Dani and Bertrand, 2007). The most common described, and most used subdivision of nAChRs in the CNS, are the homopentameric α7 nAChRs and the

heteropentameric α4β2 nAChRs. The former has a lower affinity for nicotine and their endogenous neurotransmitter acetylcholine (ACh) when compared to the latter (Dani and Bertrand, 2007; Albuquerque et al., 2009). Despite this traditional subdivision, multiple combinations of subunits are possible. In general, functional channels are made up of a single type of α- and a single type of β-subunit. However, there is also evidence of 'triplet' channels containing two different types of α- and a single type of β-subunit. Likewise, channels containing two types of β-subunits and one type of α-subunit also exist (Wang et al., 1996; Groot-Kormelink et al., 2001; Hogg and Bertrand, 2007).

nAChRs can be localized presynaptically, postsynaptically or nonsynaptically. Presynaptic and preterminal nAChRs function to enhance neurotransmitter release, the postsynaptic nAChRs contribute to fast excitatory transmission, and the nonsynaptic nAChRs are able to influence neuronal excitability by means of modulating many neurotransmitter systems (Dani and Bertrand, 2006).

Homopentameric nAChRs can be comprised of either the α7- or the α9-subunits (Hogg and Bertrand, 2007). Although, there is evidence that the α9-subunits show a preference to co-assemble with α10-subunits to form a heteropentameric α9α10 nAChR (Sgard et al., 2002). The ACh binding domain of the nAChR is at the interface between the α-subunit and the neighboring subunit (Arias, 2000), leading to as many as five functional agonist binding sites for the homopentameric nAChRs. With exception of only α-subunit containing heteropentameric nAChRs (like the α9α10 nAChR), combined α- and β-subunit heteropentameric nAChRs usually exhibit only two agonist binding sites (Taly et al., 2009). Accordingly, nAChRs generally require occupancy of two agonist binding sites (by ACh or another agonist) to achieve channel opening (Cachelin and Rust, 1994; Prickaerts et al., 2012).

Decline, disruption or alterations of nAChR function in specific cell types is believed to contribute to neurological and psychiatric disorders like for example schizophrenia, autism, Parkinson's disease, epilepsy, dementia with Lewy bodies and AD (Dani and Bertrand, 2006). As a result, these receptors have become important therapeutic targets for cognition enhancement and even pathology reduction (Toyohara and Hashimoto, 2010).

α7 nAChRs

α7 nAChRs are Ca^{2+} permeable ligand-gated ion channels expressed primarily in the brain. Like already stated, five identical transmembrane α7-subunits

surround a central channel to form this receptor. α7 nAChRs are located both pre- and postsynaptically and modulate the release of glutamate (Dickinson et al., 2008, Molas and Dierssen, 2014), GABA (Arias et al., 2010) and dopamine (Quarta et al., 2009). Furthermore, α7 nAChRs are directly involved in hippocampal long-term potentiation (LTP) (Mansvelder and McGehee, 2000), the putative cellular mechanism underlying learning and memory (Bliss and Collingridge, 1993).

These receptors have been implicated in modulating cognitive functions like attention and episodic memory (Toyohara and Hashimoto, 2010). Administration of drugs that bind to the α7 nAChR has been shown to improve cognitive function in both animal (e.g. Levin and Simon, 1998) and human studies (e.g. Newhouse et al., 1997, 2004). The main cognitive improvement with these drugs relate to memory, in accordance with the high level of expression of α7 nAChRs in the frontal-cortex and hippocampus. Different α7 nAChR agonists and modulators have been investigated for their potential to improve memory and attention disorders encountered in for instance AD and schizophrenia (Toyohara and Hashimoto, 2010). Some of these drugs are currently in phase III clinical trials (e.g. EVP-6124 or encenicline). A major drawback of α7 nAChRs is that they show rapid desensitization following exposure to agonists (Picciotto et al., 2000).

Agonists, antagonists and partial agonists

Molecules which bind to a receptor and produce a biological response can be of different classes. Here, we focus on three types of these molecules which are often encountered in nAChR drug discovery studies. These classes of molecules are: full agonists (or simply, agonists), antagonists and partial agonists. These molecules, or ligands, can be described by two independent properties, the affinity for the receptor (protein-ligand binding strength) and the intrinsic activity of the ligand (the maximal potential response from the receptor upon ligand binding).

Briefly, (full) agonists, produce the maximal potential response from receptor occupation. Although it is rather difficult to precisely know the maximal response that a receptor can produce, for many receptors the endogenous neurotransmitter (ACh in the case of nAChRs) is regarded as being the full agonist, producing the maximal response. Antagonists bind to the receptor but do not elicit a response. Hence, antagonists have no intrinsic value or activity. Partial agonists are ‘weak’ agonists and produce a response which is in between

the effects an antagonist (i.e. no effect) and a full agonist can exert. When a partial agonist produces its maximal response, all receptors are occupied, however, it is not capable of producing a response as large as the maximal response of a full agonist. Moreover, partial agonists have the unique ability to act both as agonists and antagonists depending on the concentration of endogenous neurotransmitter. By occupying the binding site of a receptor, partial agonists can compete with the binding of full agonists (or endogenous neurotransmitter) at the same site. This, in turn, can reduce the response to the full agonist, although this is also dependent upon the affinities and concentrations of both ligands. This way, partial agonists are able to reduce the amplitude of responses of more active agonists (and hence act as an antagonist). In addition, the actions of partial agonists have a ceiling effect which accounts for a rather large safety margin. This makes these ligands attractive drug candidates for therapeutic uses (Hogg and Bertrand, 2007). Moreover, it has been suggested that the known rapid desensitization following exposure to full agonists is attenuated following exposure to partial agonists (Prickaerts et al., 2012).

The allosteric model of ion channels

The functioning of ion channels has a rather complex nature. To describe the plausible functioning of ligand-gated ion channels, *the allosteric theory* of ion channel functioning was developed in 1965 (Monod et al., 1965) and adapted in 1967 (Karlin, 1967). This model predicts that ion channel receptors can exist in different conformational states. Furthermore, these receptors can undergo spontaneous transitions between these conformational states (Changeux and Edelstein, 2001).

To get an understanding of nAChR functioning, at least three conformational receptor states must be taken into account. Figure 1 shows the three conformational states in which a nAChR can be in. These states are: Active (A), Resting (R) and Desensitised (D). Agonists, antagonists and partial agonists display different affinities for each conformational state. Furthermore, a transition from one conformational state to the other depends largely upon the presence of a ligand. When no agonist is present, the receptor equilibrium is in favor of the resting (R) or closed conformational state. The receptor will stabilize within a particular conformational state in the presence of a ligand with a high affinity for that particular conformational state (Hogg and Betrand, 2007).

For (full) agonists, antagonists and partial agonists this model predicts the following (see also Figure 1):

- Agonists have a high affinity for the active (A) conformational state. Upon binding, agonists will stabilize the open conformational state of the nAChR channel. This will transiently open the ion channel pore before closing back to a resting state or to a desensitized state that is unresponsive to agonists (Dani, 2001). As the concentration of agonist is increased, the channel will be in the active/open state for a longer period of time (Hogg and Betrand, 2007).
- Antagonists show a high affinity for either the resting (R) or the desensitised (D) conformational state. Therefore, antagonist binding will stabilize the receptor in one of these closed states (Hogg and Betrand, 2007).
- Partial agonists exhibit a slightly higher affinity for the active (A) over the resting (R) or desensitised (D) conformational states. Therefore, upon binding of a partial agonist, a smaller number of receptors will be stabilized in the active state when compared to binding of a full agonist. At the single channel level, this means that partial agonist binding will stabilize the receptor in the active state for a shorter period of time, and hence evoke a smaller response of the receptor. In addition, a partial agonist has a higher affinity for closed states (R and D) than a full agonist. This explains the antagonistic effect when compared to a full agonist (Hogg and Betrand, 2007).

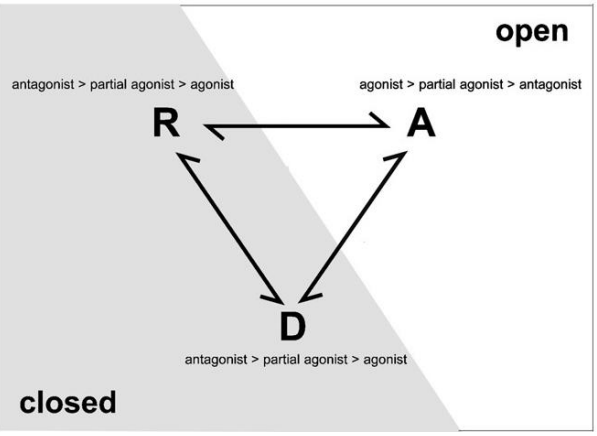


Figure 1. The minimal allosteric model for a desensitizing ligand-gated ion channel. Letters represent the active (A), resting (R) and desensitized (D) conformational states of the receptor. The receptor is closed in the R and D states. In the A state the ion channel is open and hence able to exert a biological response. The relative order of the affinities of agonists, antagonists and partial agonists of each conformational state is indicated. (Adapted from: Hogg and Betrand, 2007).

This shows that the duration of channel opening is not only dependent on agonist concentration. The distributions of open and closed conformational states of these receptors are also dependent on the individual ligand. Each ligand can evoke its own unique pattern of response. Indeed, partial agonists evoke shorter and fewer channel openings when compared full agonists (Pallotta, 1991; Hogg and Betrand, 2007).

Aim and outline of this dissertation

The aim of this dissertation was to investigate the role of α7 nAChRs in memory processes. α7 nAChR functioning was assessed on a behavioral and mechanical level in order to evaluate different possibilities to utilize this receptor subtype for treatment possibilities in different psychiatric and neurological disorders.

First, Chapter 2, 3 and 4 give a complete overview of the memory processes which were studied and the methodologies that were used to study them. **Chapter 2** provides a comprehensive overview of the existing literature on memory functioning. Specifically, this chapter contains a brief historical overview of memory research as well as current theories and associated neuroanatomy. Human as well as animal studies are discussed, resulting in a unifying framework of recognition and recall. **Chapter 3** deals with the question how to reliably measure memory processes in rodent models. This is of tremendous importance in order to be able to effectively perform preclinical research to find memory enhancing drugs. This chapter focuses on the memory test which was employed in several studies in this dissertation, the object recognition task (ORT). **Chapter 4** focuses on how memory deficits as encountered in different psychiatric and neurological disorders can be modelled in rodents. Pharmacological deficit, aging as well as transgenic rodent models will be described. Furthermore the face, construct and predictive validities of these different models will be discussed.

The following chapters will focus on cognition enhancement. The role of α7 nAChRs in memory processes was investigated in rodents. Several studies were performed in order to further increase our understanding of the role these receptors, and their different ligands, play in enhancing different memory processes. **Chapter 5** contains an experimental study in which the mechanistic and behavioral effects of a novel partial α7 nAChR agonist (EVP-6124 or encenicline) were investigated. The concept of co-agonism with existing treatments is elaborated upon in this chapter. In **Chapter 6**, a study is described in which the (sub)chronic effects of this novel partial α7 nAChR agonist were

investigated. Here the objective was to assess whether tolerance to the behavioral effect, which is suggestive of receptor desensitization, for this specific drug developed. In **Chapter 7** an experimental preclinical pharmacological model for cognitive impairments associated with schizophrenia (CIAS) was assessed. The effects of a full α7 nAChR agonist (PHA 568487), as well as two mainstay treatments (the atypical antipsychotic risperidone and the achetylcholinesterase inhibitor donepezil), were tested in this experimental paradigm.

Chapter 8 describes both mechanistic and behavioral studies to investigate an alternative strategy to enhance memory performance by utilizing α7 nAChRs. In this chapter it is shown that the effects of α7 nAChR antagonists can have two sides. On one side is the well-known, and often exploited, ability of α7 nAChR antagonists to induce cognitive deficits in rodent models. On the other side, we showed that low dose administration of α7 nAChR antagonists has a memory enhancing effect in a rodent test for memory performance. Furthermore, this data is supported by electrophysiological and hippocampal microdialysis studies where the effects of α7 nAChR antagonists were assessed. This could shed a new light upon the allosteric theory of ion channel functioning.

Finally, in **Chapter 9**, the main findings of this dissertation will be summarized and discussed after which the conclusions based on the studies presented will be provided.

References

Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., Rogers, S. W. (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiological Reviews, 89*(1), 73-120.

Arias, H.R. (2000). Localization of agonists and competitive antagonist binding sites on nicotinic acetylcholine receptors. *Neurochemistry International, 36*(7), 595-645.

Arias, H. R., Feuerbach, D., Bhumireddy, P., Ortells, M. O. (2010). Inhibitory mechanisms and binding site location for serotonin selective reuptake inhibitors on nicotinic acetylcholine receptors. *The International Journal of Biochemistry & Cell biology, 42*(5), 712-724.

Bliss, T. V., and Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature 361*, 31-39.

Cachelin, A. B., and Rust, G. (1994). Unusual pharmacology of (+)-tubocurarine with rat neuronal nicotinic acetylcholine receptors containing beta 4 subunits. *Molecular Pharmacology, 46*(6), 1168-1174.

Cascio, M. (2004). Structure and function of the glycine receptor and related nicotinicoid receptors. *Journal of Biological Chemistry, 279*, 19383-19386.

Changeux, J., and Edelstein, S. J. (2001). Allosteric mechanisms in normal and pathological nicotinic acetylcholine receptors. *Current Opinion in Neurobiology, 11*(3), 369-377.

Dani, J. A. (2001). Overview of Nicotinic Receptors and Their Roles in the Central Nervous System. *Biological Psychiatry, 49*(3), 166-174.

Dani, J. A., and Bertrand, D. (2006). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews Pharmacology and Toxicology, 47*, 699-729.

Dani, J. A., and Bertrand, D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews Pharmacology and Toxicology, 47*, 699-729.

Dickinson, J. A., Kew, J. N., Wonnacott, S. (2008). Presynaptic α7-and β2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. *Molecular Pharmacology, 74*(2), 348-359.

Dodart, J. C., Mathis, C., and Ungerer, A. (1997). Scopolamine-induced deficits in a two-trial object recognition task in mice. *Neuroreport, 8*(5), 1173-1178.

Ennaceur, A., Cavoy, A., Costa, J. C., and Delacour, J. (1989). A new one-trial test for neurobiological studies of memory in rats. II: Effects of piracetam and pramiracetam. *Behavioural Brain Research, 33*(2), 197-207.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31(1)*, 47-59.

Froestl, W., Muhs, A., Pfeifer, A. (2012). Cognitive enhancers (Nootropics). Part 1: Drugs interacting with receptors. *Journal of Alzheimer's Disease, 32*, 793-887.

Groot-Kormelink, P. J., Boorman, J. P., Sivilotti, L. G. (2001). Formation of functional α3β4α5 human neuronal nicotinic receptors in Xenopus oocytes: a reporter mutation approach. *British Journal of Pharmacology,134*(4), 789-796.

Hogg, R. C., and Bertrand, D. (2007). Partial agonists as therapeutic agents at neuronal nicotinic acetylcholine receptors. *Biochemical Pharmacology, 73*, 459-468.

Karlin, A. (1967). On the application of ''a plausible model'' of allosteric proteins to the receptor for acetylcholine. *Journal of Theoretical Biology, 16*(2), 306-320.

Levin, E. D., McClernon, F. J., Rezvani, A. H. (2006). Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology, 184*(3-4), 523-539.

Levin, E. D., and Simon, B. B. (1998). Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology, 138*(3), 217-230.

Mansvelder, H. D., and McGehee, D. S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron, 27*(2), 349-357.

Millan, M. J., Agid, Y., Brune, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., et al. (2012). Cognitive dysfunction in psychiatric disorders: Characteristics, causes and the quest for improved therapy. *Nature Reviews Drug Discovery, 11*, 141-168.

Molas, S., and Dierssen, M. (2014). The role of nicotinic receptors in shaping and functioning of the glutamatergic system: A window into cognitive pathology. *Neuroscience & Biobehavioral Reviews, 46*(2), 315-325.

Monod, J., Wyman, J., Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology, 12*(1), 88-118.

Mumby, D. G., Glenn, M. J., Nesbitt, C., and Kyriazis, D. A. (2002). Dissociation in retrograde memory for object discriminations and object recognition in rats with perirhinal cortex damage. *Behavioural Brain Research, 132*(2), 215-226.

Nehlig, A. (2010). Is caffeine a cognitive enhancer? Journal of Alzheimer's Disease, 20(Suppl 1), 85-94.

Newhouse, P., Potter, A., Levin, E. (1997). Nicotinic system involvement in Alzheimer's and Parkinson's diseases. Implications for therapeutics. *Drugs & Aging, 11*(3), 206-228.

Newhouse, P. A., Potter, A., Singh, A. (2004). Effects of nicotinic stimulation on cognitive performance. *Current Opinion in Pharmacology, 4*(1), 36-46.

Norman, G., Brooks, S. P., Hennebry, G. M., Eacott, M. J., and Little, H. J. (2002). Nimodipine prevents scopolamine-induced impairments in object recognition. *Journal of Psychopharmacology, 16*(2), 153-161.

Pallotta, B. S. (1991). Single ion channel's view of classical receptor theory. *FASEB Journal, 5*(7), 2035-2043.

Picciotto, M. R., Caldarone, B. J., King, S. L., Zachariou, V. (2000). Nicotinic receptors in the brain: links between molecular biology and behavior. *Neuropsychopharmacology, 22*, 451-465.

Prickaerts, J., de Vente, J., Honig, W., Steinbusch, H. W. M., and Blokland, A. (2002a). cGMP, but not cAMP, in rat hippocampus is involved in early stages of object memory consolidation. *European Journal of Pharmacology, 436*(1-2), 83-87.

Prickaerts, J., van Staveren, W. C. G., Şik, A., Markerink-van Ittersum, M., Niewohner, U., van der Staay, F. J., et al. (2002b). Effects of two selective phosphodiesterase type 5 inhibitors, sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP levels in the rat. *Neuroscience, 113*(2), 351-361.

Prickaerts, J., van Goethem, N. P., Chesworth, R., Shapiro, G., Boess, F. G., Methfessel, C., et al. (2012). EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacoly, 62*(2), 1099-1110.

Quarta, D., Naylor, C. G., Barik, J., Fernandes, C., Wonnacott, S., Stolerman, I. P. (2009). Drug discrimination and neurochemical studies in α7 null mutant mice: tests for the role of nicotinic α7 receptors in dopamine release. *Psychopharmacology, 203*(2), 399-410.

Sgard, F., Charpantier, E., Bertrand, S., Walker, N., Caput, D., Graham, D., et al. (2002). A novel human nicotinic receptor subunit, α10, that confers functionality to the α9-subunit. *Molecular Pharmacology, 61*(1), 150-159.

Taly, A., Corringer, P., Guedin, D., Lestage, P., Changeux, J. (2009). Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system. *Nature Reviews Drug Discovery, 8*(9), 733-750.

Toyohara, J., and Hashimoto, K. (2010). α7 nicotinic receptor agonists: potential therapeutic drugs for treatment of cognitive impairments in schizophrenia and Alzheimer's disease. *The Open Medicinal Chemistry Journal, 4*, 37-56.

Vaucher, E., Fluit, P., Chishti, M. A., Westaway, D., Mount, H. T. J., and Kar, S. (2002). Object recognition memory and cholinergic parameters in mice expressing human presenilin 1 transgenes. *Experimental Neurology, 175*(2), 398-406.

Wang, F., Gerzanich, V., Wells, G. B., Anand, R., Peng, X., Keyser, K., et al. (1996). Assembly of human neuronal nicotinic receptor α5 subunits with α3, β2 and β4 subunits. *Journal of Biological Chemistry, 271,* 17656-17665.

Chapter 2

The medial temporal lobe: Toward a unifying neuropsychobiological framework of recognition and recall

Nick P. van Goethem[1], Ruud Berkers[1], Kris Rutten,
Arjan Blokland and Jos Prickaerts
Episodic Memory: Formation, Clinical Disorders and Role of Aging recall (2014).
Nova Science Publishers, Inc., 85-133

Abstract
This chapter provides an overview of the existing theories of declarative memory and the hippocampal complex. In particular, we combined theories/models of hippocampal functioning and declarative memory (i.e. the Standard Model of Consolidation, the Spatial Map Theory, the Relational Theory, the Multiple Trace Theory, the Dual-Process Theory, and the Perceptual-Mnemonic Feature Conjunction Model) into a comprehensive unified framework. It is shown that these theories cannot fully explain the findings from empirical studies that examine the role of the medial temporal lobe in declarative memory. Therefore, the goal of this chapter was to integrate the findings reported in the literature into a comprehensive unified framework of recognition and recall. This model not only considers long-term memory and the medial temporal lobe, but also its relations with the prefrontal cortex and working memory. The proposed model implies that a familiar object, odor, sound or internal thought of a stimulus can activate an episodic representation. This is reconstructed in the hippocampus by a combination of associated polymodal stimulus information such as visual object features via the perirhinal cortex, visuospatial (contextual) information via the parahippocampal cortex, and temporal information via perirhinal cortex and associative cortices. This results in an episodic recollection. For complete conscious recollection, i.e. recognition in case of a familiar stimulus or recall in case of an internal thought of a stimulus, a representation needs access to working memory in the prefrontal cortex. Subsequently, both the old as well as newly-formed explicit/declarative memory traces (in case of a novel stimulus or context) can

then be reconsolidated and consolidated, respectively. The consolidation takes place in the perirhinal and parahippocampal cortices or as semanticized memory traces in the neocortex. Finally, these memory traces can be retrieved again in subsequent recollections to dynamically form episodic memories.

Introduction

The case-study of patient H.M. proved to be the starting point of an extensive body of research on hippocampal functioning in the brain in relation to mnemonic functioning (Scoville and Milner, 1957). Surgical resection of a large part of the medial temporal lobe (MTL) of patient H.M. caused severe amnesic symptoms, while alleviating the epileptic seizures that were the reason for this drastic surgical procedure. Remarkably, although H.M. promptly suffered from extreme amnesia, his intelligence, language and attention capabilities remained largely intact. Skill learning was also found to be largely intact (Corkin, 1968), indicating the potential existence of relatively separable memory systems. Therefore, declarative memory was considered dependent on the absent hippocampal structure, while nondeclarative memories, such as procedural learning, priming, conditioning and nonassociative learning, seemed dependent on other intact structures (Cohen and Squire, 1980; Squire and Zola, 1996). Separate memory and learning systems were determined for conditioned motor responses (Thompson and Krupa, 1994) and emotional learning (LeDoux, 1993). The MTL was attributed a specialized role in declarative memory, but the functional processing and contribution of structures in the MTL are up until this point the subject of fierce controversy (Diana and Ranganath, 2011; Montaldi and Mayes, 2011; Wixted and Squire, 2011a, 2011b; Libby et al., 2012; Kim et al., 2014). Several hippocampal theories have been proposed (O'Keefe and Nadel, 1978; Squire and Alvarez, 1995; Eichenbaum, 1997; Nadel and Moscovitch, 1997). More recently, in addition to the hippocampus, the role of neighbouring cortical areas in the MTL (Aggleton and Brown, 1999), and the prefrontal cortex (PFC) (Simons and Spiers, 2003; Wang and Morris, 2010; Kim et al., 2011) have been considered in declarative memory functioning.

Declarative memory is distinct from other memory types as it concerns memories of which one is aware (explicit memories), in contrast to other procedural memory types of which one is unaware (implicit memories) (Roediger, 1990). Declarative memory can be divided into two types: episodic and semantic (Tulving, 1972). Episodic refers to contextual memory, i.e., memory of specific events or stimuli related to their temporal and spatial context. It includes sensory information, allowing one to re-experience an event. Semantic memory on the other hand, refers to non-contextual memory, i.e., general knowledge about the world around us. It refers to general concepts and their relations, categories, facts, people, events or word meanings; items that are not particularly grounded in the experience of a temporal or spatial context (Tulving, 1986). It is still not entirely clear what the role of the MTL and related brain structures is in the processing of respectively episodic and semantic memory traces and the extent to which they rely on separable memory systems.

Another subdivision in memory can be made in terms of recognition memory: the ability to judge a presented stimulus as 'old' or 'new', depending on whether it has been observed at an earlier occurrence. Recognition memory can be subdivided into two types, namely recollection and familiarity. Recollection refers to re-experiencing an event, while familiarity refers to merely having the feeling that one has perceived the stimulus before, without having this feeling grounded in a context or source. The conceptual separation of recollection and familiarity is based on evidence concerning the neural substrates of memory, implicating two separate functional networks. As we will discuss in more detail later, the Dual-Process Theory of memory considers recollection as analogous to the re-experiencing aspect of episodic memory (Aggleton and Brown, 1999). In other words, recollection, also referred to as 'remembering' (Knowlton and Squire, 1995), entails retrieving an event or stimulus in its temporal and spatial context, thereby mentally traveling back in time to relive the episode (Suddendorf and Corballis, 1997). On the other hand, familiarity refers to merely recognizing a sensory stimulus and attributing it as presented before, also referred to as 'knowing' (Knowlton and Squire, 1995). The feeling that one has seen an object or face, or heard a certain sound before is a feeling of familiarity. However, familiarity by itself is not a full-blown episodic memory, as it implies no or only partial knowledge of the contextual details of the previously experienced stimulus. An alternative to the Dual-Process theory is to conceptualize the distinction between familiarity and recollection as primarily concerned with differing memory strengths within a qualitatively similar memory system (Wais et al., 2006). Some also regard semantic memory as a form of familiarity (Henson and Gagnepain, 2010), but here we consider mostly the perceptual type as familiarity for modality-specific (sensory, lingual and motor) representations and semantic memory as consisting of amodal conceptual knowledge and categorical relations amongst concepts (Patterson et al., 2007).

This chapter provides an overview of theories/models of episodic and recognition memory (i.e. the Standard Model of Consolidation, the Spatial Map Theory, the Relational Theory, the Multiple Trace Theory, the Dual-Process Theory, and the Perceptual-Mnemonic Feature Conjunction Model) and the MTL, and synthesizes selectively reviewed results into a unifying neuropsychobiological model that not only considers long-term memory (LTM) and the MTL, but also relations with the PFC and perceptual association areas. We will also mention the role of temporal aspects in recognition and recall, and try to fit this parsimoniously within the known anatomical structures of the brain and their information processing functions, drawing upon recent ideas and controversies in the literature.

Neuroanatomy of the medial temporal Lobe

The hippocampal formation has for long been considered to be a part of the limbic system, including other structures such as the cingulate gyrus and amygdala (MacLean, 1955). On the anterior side, the hippocampus is bordered by the amygdala and on the posterior side by the isthmus of the cingulate gyrus, also called retrosplenial cortex (Kingsley et al., 1999; Andersen, 2007). On the ventral side, the hippocampal formation is bordered by the parahippocampal gyrus, consisting of the perirhinal, entorhinal and parahippocampal cortex, and reciprocal connections connect these substructures. The hippocampal formation itself consists of three parts: the dentate gyrus (DG, also referred to as CA4), the hippocampus proper (consisting of regions CA1, CA2 and CA3) and the subiculum (Kahn et al., 2008; van Strien et al., 2009). The hippocampus is bordered by the fimbria and dorsal fornix; both contain connecting fibers from the hippocampus and are often also considered part of the hippocampal formation. The fornix is the tract connecting the hippocampus with the mammillary bodies (Hunsaker et al., 2008).

The entorhinal cortex is situated in the parahippocampal gyrus, along with the parahippocampal cortex and perirhinal cortex, both believed to be the endpoint of relatively separable visual processing streams in association cortices (Mishkin et al., 1997; Kahn et al., 2008; Libby et al., 2012). The perirhinal cortex receives input from visual areas TE and TEO, which are regions of the visual association cortices in the ventral stream (Suzuki and Amaral, 1994; Ungerleider and Haxby, 1994; Mishkin et al., 1997), and polymodal sensory areas (Furtak et al., 2007). These regions of the ventral visual stream are important for visual object processing (Tsao et al., 2010). The visual areas that are part of the dorsal visual spatial stream, including posterior parietal cortex, posterior cingulate and retrosplenial cortex, project to the parahippocampal cortex (Burwell, 2000; Kahn et al., 2008; Kravitz et al., 2011). The perirhinal cortex and the parahippocampal cortex provide input through respectively the lateral and medial entorhinal cortex to subsequently converge in the subiculum and the hippocampus (Burwell, 2000; van Strien et al., 2009). Reciprocal feedback connections project back to the visual association cortices (Agster and Burwell, 2009). Additionally, the parahippocampal cortex also provides input to the perirhinal cortex. Based on this anatomical connectivity, it is postulated that the perirhinal and parahippocampal cortex are differently specialized for respectively object and spatial recognition memory (Buffalo et al., 2006). As will be discussed in more detail later, a hierarchical converging visual stream can be discerned along these different brain areas converging in the hippocampus (Lavenex and Amaral, 2000; Hoover and Vertes, 2007). Furthermore, the MTL is interconnected with the PFC via reciprocal connections. The PFC can be divided into subregions, such as anterior (APFC), dorsolateral (DLPFC), ventrolateral

(VLPFC), orbitofrontal (OFPFC) and medial (MPFC) parts (Simons and Spiers, 2003). The DLPFC and OFPFC have strong reciprocal connections with the perirhinal and entorhinal cortices (Rempel-Clower and Barbas, 2000). There are also unidirectional projections from the CA1 to the caudal region of MPFC (Barbas and Blatt, 1995).

Hippocampal memory functioning

Theories of hippocampal memory function

The standard model of consolidation, or Declarative memory theory

Since the first investigations of patient H.M., a wealth of research has accumulated on the function of the hippocampus in declarative memory. The most important finding concerning H.M.'s memory performance was his inability to form new declarative memories, a condition known as anterograde amnesia. Furthermore, H.M. was unable to retrieve memory traces of events that occurred up to two years prior to his surgery, a condition termed temporally graded retrograde amnesia (Scoville and Milner, 1957). Following these observations, the Standard Model of Consolidation was formulated (Squire et al., 1984). It postulates an a-specific time-dependent role of the hippocampus in consolidating both semantic and episodic types of declarative memories (Squire et al., 2004). According to this model, following a consolidation period, both episodic and semantic declarative memories are stored in areas outside of the hippocampus. This could explain H.M.'s limited retrograde amnesia of incompletely consolidated memories, as well as an anterograde amnesia resulting from the inability to consolidate new memories. Preserved memory for older, but not for more recent memories, is reflected in a Ribot-gradient in memory performance (Frankland and Bontempi, 2005). In this framework, the hippocampus serves as a temporary relay station for all declarative memories until they are well embedded in an extra-hippocampal network. Therefore, memory can be explained in terms of age of the memory trace.

The spatial map theory

Before the Standard Model of Consolidation, another theory was postulated; the Spatial Map Theory (O'Keefe and Nadel, 1978). This theory focuses specifically on spatial relational processing, postulating that the function of the hippocampus is the representation of events in a spatial or allocentric context. As such, the hippocampus relates various visuospatial cues to reconstruct a representation of locations in the form of a map. This map aids flexible navigation in a familiar environment from a novel starting point using the relative position on a spatial allocentric map (Burgess et al., 2002). This

allocentric map also serves to support the spatial context of episodic memories. This theory claims that the hippocampus is important for the formation of spatial relations among elements of declarative memories.

The relational theory

After this original hypothesis, alternative explanations of hippocampal functioning in memory have been proposed. The Relational Theory (Cohen and Eichenbaum, 1997) emphasizes the general role of the hippocampus in establishing flexible relations among different items, allowing for a flexible association of information stored in dispersed neocortical modules and modalities (Henke, 2010). The hippocampus allows for relational memory construction, flexibly combining different traces and allowing the information about a scene or event to be flexibly used. Without the hippocampus, retrieval of these traces is rigid. Thus, hippocampal damage would impair episodic *and* relational semantic/spatial retrieval, possibly allowing only for the rigid retrieval of isolated semantic concepts.

The multiple trace theory

Another alternative for the role of the hippocampus in forming a declarative memory trace is provided by the Multiple Trace Theory. This theory states that the hippocampus is important in the initial encoding and consolidation of all information that is attentively processed (Nadel and Moscovitch, 1997). Since the hippocampus receives information from polymodal association cortices, and combines the perceptual input from the ventral visual stream, dorsal visual stream, auditory cortices and olfactory cortices, all the information that is part of an experience is combined into a memory trace. It contains representations acting as an index of neocortical ensembles that represent perceptual and semantic information in order to complete an episodic representation (Teyler and DiScenna, 1986). According to this framework, semantic memory is mediated by a network of neocortical structures (Moscovitch et al., 2005).

When episodic memory encoding/recollection activates neocortical areas in conjunction, through repeated experience, retrieval or replay, mutual connections are formed between neocortical areas that represent abstracted semantic information without context. This enables relevant information from experiences to be extracted in neocortical networks, thus enabling consolidation in an interconnected extrahippocampal network of semantic ‘nodes’. This semantic network consists of abstract concepts in knowledge structures, or schemas, which can be retrieved independently of the hippocampus. These concepts are abstracted from sensory, experiential correlates. At the same time, the hippocampus is still required to enable episodic contextual recollection, and it does so by combining multimodal traces to enable the re-experiencing of

events. Hippocampal involvement in representing a memory trace is thus based on the vividness or experiential quality of the trace, not necessarily its age (Gilboa et al., 2004). As memory traces are 'semanticized' over time the hippocampus becomes less important for their retrieval, potentially explaining the ability of H.M. to retrieve old memory traces. These explanations of memory are different from the Standard Model of Consolidation, accounting not primarily for declarative memory substrates in terms of their age, but rather for their information content.

The dual-process theory

The difference between remembering (or recollection of) a stimulus or event in its spatial and temporal context and merely having the feeling that one has seen a stimulus or event before (i.e. familiarity) is not addressed by the above mentioned theories. However, it is open for discussion whether individual sensory components contributing to perceptual familiarity are also represented by the hippocampus, or whether the hippocampus functions merely as a information-'binder', or as an 'index' for the reactivation of individual perceptual association areas during re-experiencing. The Dual-Process Theory differentiates between recollection and familiarity. Familiarity-type recognition occurs if a word, picture or face is merely recognized as seen before, whereas in recollection, the recognition can be put into a context, i.e., when and where it was seen. The Dual-Process Theory of memory views recollection as analogous to episodic memory; remembering an event or stimulus in the context of its temporal and spatial dimension (Aggleton and Brown, 2006). On the other hand, familiarity is a separate perceptual-based memory type which does not contain a spatial and temporal context.

As will be discussed in detail in the following sections, separate memory systems have been proposed which might indeed be conceptually separable to some degree. According to the Dual-Process Theory, the recollection-system comprises the extended hippocampal system, including hippocampal connections to the mammillary bodies and anterior thalamic nuclei, while the familiarity-based system is located in the perirhinal cortex and its connections to the dorsomedial nucleus of the thalamus (Aggleton and Brown, 1999). The alternative interpretation of the familiarity/recollection-distinction is that recognition memory is equally subserved by the hippocampus and the perirhinal cortex, implying that no qualitative distinction exists between familiarity and recollection (Wixted and Squire, 2011b). Following this argument, dissociations between familiarity and recollection merely reflect a distinction between strong and weak memories, instead of a qualitative distinction of memory processes.

Studies of hippocampal memory function

Research

The first line of studies on hippocampal declarative memory function consists of neuropsychological data from human lesion studies. These studies have shown that damage to the MTL impairs mostly detailed episodic memories regardless of their age, whereas (temporarily graded) semantic retrograde amnesia can also often be found up to ten years back, but only very inconsistently for older memories (Fuji et al., 2000). Thus, a robust time-independent deficit after lesions to the MTL was found in tasks requiring recall or re-experiencing of an event with episodic detail, but not for retrieving semantic concepts (Lah and Miller, 2008). Along similar lines, remote semantic memories are likely preserved in case of a hippocampal lesion, whereas all episodic detailed memories are affected by hippocampal lesions regardless of their age. Indeed, it has been shown that two patients with extensive MTL damage were still able to acquire semantic knowledge and when asked to retrieve this information, they were able to provide additional information about these concepts showing that these patients are able to develop new semantic relations (Bayley et al., 2008). This is incongruent with a Standard Model of Consolidation, arguing instead that hippocampal involvement is essential for episodic memory and exerts at most a nonessential, temporary influence on the formation of semantic knowledge.

The Spatial Map Theory assumes a central role for the hippocampus in forming a cognitive and spatial map representing allocentric space, thus providing a spatial context into which disperse elements of an episodic experience can be combined (Kumaran and Maguire, 2005). However, amnesic patient E.P. suffered from complete bilateral damage to the MTL (Teng and Squire, 1999), but was able to recall the spatial layout of the region he grew up in and moved away from more than 50 years ago. Patient H.M. also had a preserved ability to memorize new spatial layouts or recognize complex pictures (Corkin, 2002). The study of patient K.C. revealed that despite bilateral hippocampal lesions, he was able to perform normally on allocentric spatial tests of his neighbourhood and the world. He was however impaired on recalling specific location details, indicating this deficit was unrelated to spatial representation per se, but specifically related to episodic recall of spatial details (Rosenbaum et al., 2000). This evidence suggests that the hippocampus is not necessarily required for all representations of allocentric space, but that the hippocampal processing of information in allocentric space merely serves to provide an accurate spatial index to experiential representations.

Hippocampus

A second line of studies on hippocampal declarative memory function use imaging techniques. Thus, it was shown that retrieval of recent and remote

detailed autobiographical memories seem to activate the hippocampus equally (Cabeza and St Jacques, 2007), which is in contrast with predictions of a lower hippocampal involvement for remote memories. The hippocampus and neocortex appear to be equally activated when participants were recollecting detailed autobiographical memories that were less than four years old and memories that were more than twenty years old (Ryan et al., 2001). Other activated regions during recollection are the parahippocampal gyrus, PFC, temperoparietal junction, posterior cingulate and precuneus gyrus (Rekkas and Constable, 2005). The finding of relative time-independent hippocampal activation in response to remote and recent detailed autobiographical memories has subsequently been replicated for memories matched in terms of vividness and personal significance (Söderlund et al., 2011). Therefore, vividness, or amount of episodic time/place detail, of a memory trace might be confounded with memory age, as memory traces transform with age, except for the important memory traces of individual events (Winocur et al., 2007; Winocur and Moscovitch, 2011). In addition, information that is worth keeping contained in episodic traces is likely abstracted over time in neocortical semantic networks or schemas (Nadel and Moscovitch, 1997; Kan et al., 2009). This process is also known as systems consolidation (Wiltgen and Tanaka, 2013).

One fMRI-study compared spatial semantic and spatial episodic memories of different ages at retrieval. The hippocampus was found to be preferentially activated when participants recalled episodic spatial information, compared to when they recalled semantic spatial information (Mayes et al., 2004). The hippocampus is thus preferentially activated in response to primarily episodic memory retrieval, and concurrently to the spatial aspects of memory retrieval, but hardly to nonspatial semantic memory retrieval (Hoscheidt et al., 2010). Similarly, it was also found that the hippocampus is increasingly activated in response to tasks probing the retrieval of episodic relations compared to semantic relations and with the retrieval of spatial relations compared to nonspatial relations (Ryan et al., 2010).

A seminal study examining hippocampal activation in spatial tasks is the study of London taxi drivers (Maguire et al., 1997). In this study, Positron-Emission Tomography (PET) was used to determine activation in experienced taxi drivers in London while they navigated complex routes across the city. This study found activation in the right hippocampus, parietal, parahippocampal, posterior cingulate cortex, and precuneus in relation to the spatial content of a memory. In another study, participants were scanned to determine the activated brain structures in various tasks concerning the spatial organization of Toronto (Toronto Public Places Test). Activation was found in the MTL, but not in the hippocampus proper (Rosenbaum et al., 2004). The activation of different

extrahippocampal regions varied with different task requirements. The retrosplenial cortex was activated in tasks that involved distance and proximity judgements in allocentric space, whereas the superior-medial parietal cortex was implicated in egocentric tests that involved imagined movement like landmark sequencing. Familiarity-related memory for places was related to parts of the anterior temporal cortex, whereas the parahippocampal gyrus was activated in response to all spatial tasks (Rosenbaum et al., 2004). A similar study found activation in the same regions during a city navigation-task (Kumaran and Maguire, 2005). Strikingly, the hippocampus does not appear to be involved when navigating through a highly familiar spatial environment (Hirshhorn et al., 2011).

Taken together, irrespective of the different hypotheses of hippocampal memory function, the human lesion and imaging data do indicate that the hippocampus is clearly involved in both consolidation and retrieval of episodic memories. In the following sections we will discuss studies which investigated how recognition is implemented in episodic memory function, while also focusing on recall and semantic memory.

Recognition memory: familiarity and recollection

Recognition memory performance

Behavioral performance in recognition memory tasks is often explained using signal detection theory. In subsequent memory tasks, subjects are repeatedly presented with a stimulus list and after a delay they judge whether subsequently presented test stimuli are the same or different from those on the list (Yonelinas, 2001; Squire et al., 2007). Subsequently, hit and false alarm rates are measured to construct Receiver-Operator-Characteristics, or ROC, curves. Same/different judgments are made along a background of varying confidence levels and response biases that influence the likely behavioral response in cases of uncertainty. The shape of the ROC-curves plot *hit rates* (correctly recognized) versus *false-alarm rates* (incorrectly detected), which vary across these response biases and confidence levels. The curves could have a completely curvilinear and symmetrical shape (see right panel in Figure 1). Alternatively the curve could have a positive intercept, causing the curve to be asymmetrical (see left panel in Figure 1). The presence of these different curves is often explained in two different ways. According to Dual-Process Theory, the y-intercept reflects a threshold for recollection, which is assumed to be an all-or-none process where false alarms do not occur (Yonelinas, 2001).

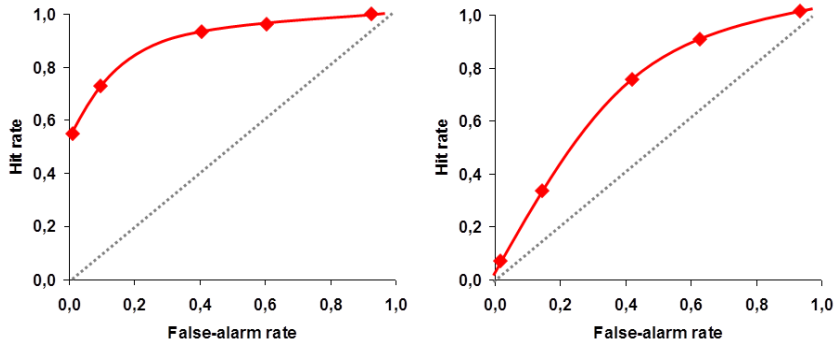


Figure 1. ROC-curves displaying recognition performance as mediated by either familiarity or familiarity and recollection. ROC-curves are plotted when recognition occurs by means of A) both recollection (as seen from the y-intercept) and familiarity (as seen from the curvilinear shape, or B) familiarity alone (as seen from the absence of a positive y-intercept).

In recollection, the event is re-experienced upon stimulus presentation, and confidence and accuracy of the judgment is automatically high. The curvilinear shape reflects familiarity-typed memory, with differing degrees of confidence and response biases that influence decisions in cases of uncertainty, and therefore influence different accuracies and the occurrence for false alarms. Recollection judgments are confident, while the sense of familiarity with an object varies in strength (Yonelinas, 2002). The alternative interpretation of these different curves considers them as only distinguishing between weaker and stronger memories and qualitatively similar memory signals. According to this interpretation, the difference between recollection and familiarity is a matter of differing sensitivities and specificities with differing memory strengths (Squire et al., 2007; Wixted and Squire, 2011b).

Human studies

In patients with selective hippocampal damage, dissociations can be found between the ability to spontaneously recall information and the ability to merely recognize presented information. Patients with transient hypoxic lesions of the hippocampus are found to display selective recollection-deficits, as measured by patients' self-reporting (indicating whether they "Remember" or "Know" an item) and an analysis of ROC-curves. Patients with lesions that also extend to the parahippocampal gyrus display additional deficits in familiarity (Yonelinas et al., 2002). This suggests recollection and familiarity might be a dual-process, depending on dissociable anatomical substrates. Another study revealed that both patients with selective hippocampal damage and controls displayed a shift from asymmetric to symmetric ROC-curves of subsequent memory performance, equated with a shift of general memory strength. When

memory strength was matched, both patients and controls displayed a symmetric ROC-curve (Wais et al., 2006), indicating a general weakening of the memory trace in the hippocampus instead of a qualitative shift in neurological and cognitive substrates (Squire et al., 2007), thus arguing against the Dual-Process Theory.

Event-related fMRI-measurements during memory retrieval of a certain learned item have shown that hippocampal activity only increases when old items were recollected, not when items were labeled 'new' or merely 'familiar' (Eldridge et al., 2000). Another fMRI-study found activation in the perirhinal cortex correlated with the level of recognition confidence during a familiarity judgment, while the hippocampus was found to be only activated in case of a recollection judgment (Montaldi et al., 2006). Another study measured no perirhinal cortex activation, and hippocampal activation during recollection was inversely correlated with the strength of familiarity-based memory (Yonelinas et al., 2005). Again another study found dissociations in the MTL between familiarity, recollection and novelty. All-or-none recollection at retrieval was correlated with activation of the posterior hippocampus. Activation of the parahippocampal cortex and anterior hippocampus was positively correlated with the degree of familiarity or confidence of the subject. In contrast, the perirhinal and entorhinal cortex were negatively correlated with degree of familiarity and seem to be activated in response to novelty. Even though these results vary, these studies do provide evidence for a separable characterization of recollection and familiarity-processes during retrieval. They point in the direction of a dual-process interpretation (Daselaar et al., 2006).

The perirhinal cortex is probably important in detecting the novelty of stimuli, for example by comparing a stimulus with stored stimuli. On the other hand, the hippocampus can be linked to detecting novel contextual stimuli; i.e., the detection of a stimulus in an unexpected context (Eichenbaum, 1999; Nyberg, 2005). The hippocampus is then probably required for the detection of new associative and contextual stimuli, thereby mediating recollection, while the perirhinal cortex is important for the detection of new single stimuli, thereby mediating familiarity (Nyberg, 2005). It has been argued that the rhinal cortex functions as a 'gatekeeper' for declarative memory formation in the hippocampus, aiding the preferential encoding of information in the hippocampus on the basis of the presence of a novel episodic element (Fernández and Tendolkar, 2006). In this sense, the rhinal cortex is involved in detecting novelty and familiarity of a stimulus, coaxing the encoding of a new episodic memory trace by the hippocampus.

The dissociation between levels of activation of both perirhinal cortex and hippocampus found at retrieval of recollective experiences should then not necessarily exist at the time of initial encoding of a novel experience. Indeed, activity at encoding in both the hippocampus and perirhinal cortex have been found to predict successful subsequent recollection of items (Shrager et al., 2008). It is reasonable to postulate that the perirhinal cortex does aid in the formation of episodic memories, by signaling novelty of an experience. This explains why both are equally activated during the encoding of a recollective trace. During retrieval, recollective memories require the reinstatement of activity in both the hippocampus and neocortical areas possibly as a result of the detection of familiar objects by the perirhinal cortex. The mere signaling of novelty/familiarity necessitates only the perirhinal cortex, allowing the feeling of familiarity to be preserved in case of hippocampal damage (Sauvage et al., 2008).

The dissociation between remembering and knowing is neither absolute nor a double dissociation, it is likely that both contribute to recollection (Davachi, 2006). In the case of recollection, the hippocampus has a focus on completing episodic representations, combining domain-specific input from nearby cortical areas. Specifically, input from the perirhinal cortex for object features, and from the parahippocampal cortex for spatial/contextual features (Diana et al., 2007). The rhinal cortices can signal novelty/familiarity independently, but the hippocampus is additionally recruited for the encoding and retrieval of a recollected event by combining this domain-specific input. The debate between Dual-Process Theory and memory strength theory remains controversial (Diana and Ranganath, 2011; Montaldi and Mayes, 2011; Wixted and Squire, 2011a, 2011b), but both theories provide a somewhat simplified account. Familiarity and recollection seem to be dissociable singularly, in the sense that familiarity can occur without recollection, but recollection probably also recruits familiarity-signals.

An alternative take on this issue has been to argue that activation of the perirhinal cortex is invoked to support the familiarity and novelty-detection of not only single items or objects, but also a pair of items if they have been processed as a single configuration. Here, the feeling of familiarity pertains to a configuration of stimuli. According to this ‘Unitization’-hypothesis, regions outside the hippocampus, notably in the rhinal cortex, can support recognition of associative scenes, if they have been encoded as a single compound stimulus (Haskins et al., 2008). This hypothesis was corroborated by studies that have promoted the unitized encoding of an item and source information (e.g. an object and a background color). In one such study, participants were asked to rate the plausibility of an item to occur against the context of a background

color, promoting the unitized encoding of these two items. In other encoding trials, the task was unrelated to unitizing item and context (e.g. making size or pleasantness judgments depending on the background color). The unitized encoding condition indeed promoted a more curvilinear shape of the ROC-curve at retrieval (Diana et al., 2008), reflecting an increase in familiarity. Similarly, using an unitization-approach where two words are either encoded as separate lexical units in sentences or as unitized compounds, hypoxic patients with recollective deficits show an advantage of recognizing the unitized word pairs over the non-unitized pairs (Quamme et al., 2007). This was confirmed in a fMRI-study that found that both perirhinal cortex and hippocampal activation were related to accurate source recognition when a unitization-encoding condition was employed (Staresina and Davachi, 2009).

Animal studies

In rodents and primates, circumscribed brain damage can be induced for the experimental assessment of subsequent cognitive deficits. By using an experimental assessment, it might be possible to find dissociations of brain regions and brain processes. Of note, the DLPFC in primates corresponds to the MPFC in rodents, likewise the counterpart of the parahippocampal cortex in primates is the postrhinal cortex in rodents (Uylings et al., 2003). It has to be realized that these differences between species might have functional consequences.

Examples of memory paradigms used in animals are delayed matching to sample (DMS) or delayed non-matching to sample (DNMS) paradigms (Otto and Eichenbaum, 1992; Squire et al., 2007). Here, in a training phase, rats or monkeys are repeatedly presented with a stimulus and their response to this stimulus is rewarded. This results in stimulus-response learning. In subsequent trials, the same stimulus is presented together with a new stimulus. In this phase, animals are either rewarded for responding to the new (DNMS) or the old (DMS) stimulus or object. After the matching or non-matching rule has been learned up to criterion, performance is assessed by the ratio of correct/incorrect choices over successive trials which can again be plotted in ROC-curves.

The DNMS paradigm has been applied to an odor recognition task (Fortin et al., 2004). Here, rats are required to dig into a cup of sand for a food reward if the scent paired with the cup is different from a scent presented earlier. Alternatively, when the scent has been presented previously in combination with the cup, they should refrain from digging, and approach an alternative empty cup in the back. After being trained up to an 80 % correct criterion within five training trials, the ROC-curves of memory performance can be obtained. It

was found that the ROC-curves for rats with hippocampal lesions are symmetric and entirely curvilinear. According to the Dual-Process Theory, the removal of the y-intercept on the ROC-curve indicates that hippocampal damage impairs recollection-typed memory. The curvilinear symmetrical shape that remains after hippocampal lesions indicates a preservation of familiarity-based memory. Familiarity-based memory should therefore be dependent upon an alternative memory structure, in line with a dual process.

Another paradigm often used in rodent research is the spontaneous object recognition task (ORT) (Ennaceur and Delacour, 1988; Ennaceur, 2010; Akkerman et al., 2012; van Goethem et al., 2012). In this task, the natural tendency of animals to explore novel objects is used to assess their ability to recognize an object. During a training period, the rat is presented with two objects, and after a delay period, the rat is presented with one of the old objects and a new object. The rat is believed to recognize the old object if it significantly spends more time exploring the novel object. The ORT can be extended to test associative recognition by presenting the rat with multiple object manipulations (for instance: location and odor) and allowing the rat to distinguish between new and old stimulus pairings in the recognition-phase. In an odor recognition paradigm, the exploratory preference of pairings between the odor and storage medium where the reward was hidden can be discerned. Thus, it was shown that in control rats, the ROC-curve is asymmetrical and linear, meaning that discrimination performance was primarily based on recollection of the specific association. After hippocampal lesions, the ROC curve changed into a curvilinear symmetrical familiarity curve (Sauvage et al., 2008). Hence, a shift in ROC-curves occurred, indicating a move from recollection to familiarity after hippocampal lesions, again suggesting that familiarity and recollection have different anatomical substrates. The dissociation shown is not a double dissociation, because the preservation of recollection in the light of familiarity-deficits due to rhinal lesions is not a replicated observation (but see: Bowles et al., 2007), and are less likely to occur as familiarity probably contributes to recollection.

A sophisticated manipulation of the ORT was used in a one-trial exploration paradigm manipulating both object recency (temporal manipulation) and object location (spatial manipulation) in an arrangement of four objects (Li and Chao, 2008). Both controls, sham lesioned, and rats with an electrolytic lesion of dorsal region CA3 were able to perform a radial arm-maze task where rats preferentially explore objects in new locations, temporal order ORT where rats preferentially explore objects that were presented longer before in a succession, and classical ORT. However, when both location and temporal presentation of objects were manipulated at the same time, a performance-interaction emerged in the control and sham lesion rats which was absent in the lesioned

rats. Lesioned rats appeared to perform differently in tasks that require the integration of what-where-when information, whereas tasks that depend only on processing what, where and when-information individually can be performed without CA3-involvement. The CA3 thus appears to be involved in the joint encoding and retrieval of object, spatial and temporal information (Li and Chao, 2008).

The response of a brain region in memory retrieval tasks can also be assessed using single-cell electrophysiological recording of local neuronal firing or spiking. The repeated presentation of stimuli is related to a decrease in neuronal response in the anterior inferior temporal cortex; specifically the perirhinal cortex of monkeys. In this region, a large majority of the neurons measured (i.e., 98 %) displayed a decrease in signaling in response to familiar stimuli. These findings indicate that the perirhinal cortex does not fire in response to familiarity (Xiang and Brown, 1998). This decrease in neuronal firing might provide the information necessary for judgment of previous occurrence. Similarly an increase in neuronal firing might signal novelty. The next section will explore in more detail the involvement of the rhinal cortices and the hippocampus in both familiarity and recollection, but also on perception of object, spatial and temporal information.

Object and context recognition

Object perception and recognition

Studies in rats showed that lesioning the hippocampus has no effect on the ability to recognize objects, while lesioning structures surrounding the hippocampus, i.e., the parahippocampal gyrus, does impaire the ability to recognize objects (Mumby, 2001). Distinct roles for the hippocampus and perirhinal cortex have been found as well in object recognition abilities (Kim et al., 2014). For example, rats improve on the spontaneous ORT when the initial exploration phase is extended from 4 to 8 min. However, rats with a lesioned perirhinal cortex do not improve, and the extent of damage to the perirhinal cortex is positively correlated with their deficits on recognition memory (Albasser et al., 2009) highlighting the importance of the perirhinal cortex in recognizing objects. A method to investigate the importance of the perirhinal cortex is to infuse the sodium channel blocker lidocaine at different stages of the spontaneous ORT. Lidocaine temporarily inhibits activity in a region allowing for a direct test of the involvement of the perirhinal cortex in recognition memory. Lidocaine administration was thus found to be detrimental to performance in all phases of the ORT, i.e. acquisition, consolidation and retrieval

memory processes. These results indicate the perirhinal cortex is important in all of these processing stages (Winters and Bussey, 2005).

The Perceptual-Mnemonic Feature Conjunction Model is yet another model concerning declarative memory. It is aberrant from the other herein described theories in that it is not a theory of hippocampal memory function. Instead, it tries to explain the role of the perirhinal cortex by viewing it as the locus of the representation of conjunctions of visual object features (Bussey and Saksida, 2002). Here, individual features, represented by regions of the ventral visual stream, converge into one representation of the object. This configural stimulus is, perhaps temporarily, stored in the perirhinal cortex. The perirhinal cortex is therefore required for resolving feature ambiguity in complex object identification, as it is the first place where multiple features are processed in one configuration. Stimuli with feature ambiguity are defined as stimuli that cannot be distinguished on the basis of single features alone, only on the basis of the configuration of features. By retrieving stored traces of presented compound stimuli, feature ambiguous stimuli can be discerned in familiarity-based memory. Lesions of the perirhinal cortex should therefore impair this type of memory (Cowell et al., 2006).

The predictions of this model are twofold, namely that both discriminating different complex objects, as well as recognizing these objects, crucially depend on the perirhinal cortex. Based on observations from earlier studies, three features of the functioning of visual recognition memory were tested (Cowell et al., 2006), namely that there should be an increase in object recognition impairment due to lesioning the perirhinal cortex after: i) increasing the delay between presentation-phase and test-phase (Meunier et al., 1993; Mumby and Pinel, 1994), ii) increasing the number of objects to be remembered (Buckley and Gaffan, 1998), and iii) using a large number of stimuli ensuring that each stimulus is trial unique instead of repeatedly presented across training trials (Eacott et al., 1994). Simulating lesions of the perirhinal cortex in a neuronal network computer model of the ventral visual stream confirmed these predictions (Cowell et al., 2006): a deficit in object recognition increased with larger delays between stimulus-presentation and testing. An explanation for this effect is the interference among information represented by regions other than the perirhinal cortex that occurs in the delay-period by input of other visual stimuli. Interference does not cause an equivalent amount of forgetting with an intact perirhinal cortex. The representations of unique configurations of features are more resistant to interference, because compound objects are more unique and less likely to be presented repeatedly than single visual features. The perirhinal cortex is therefore valuable in object recognition because it can detect novel stimuli as novel configurations of visual features,

making recognition robust to interference. Similarly, longer lists of the objects to be remembered or using trial-unique stimuli leads to an impaired detection of novel stimuli when the perirhinal cortex is lesioned, because of an increased chance of interference of object features. The perirhinal cortex can thus be considered as the node of convergence of different visual features, and as the necessary area for resolving feature ambiguity.

Monkeys with perirhinal cortex lesions are also impaired in the performance of visual discrimination of objects with a high level of feature ambiguity. For example, in one study monkeys were taught to touch one odd object among similar objects on a touch screen. When the odd object differed from the other images only on colour, shape or size, monkeys with lesioned perirhinal cortices performed just as well as controls. However, when the odd object had to be picked out from three similar objects that were seen from different angles, or similar objects that were presented against the background of a differing unfamiliar context, the monkeys were impaired (Buckley and Gaffan, 1998). Also, two distinct images can be blended to create morphed images that are presented on a computer screen, and monkeys with perirhinal lesions were found to be impaired in discriminating these images (Bussey et al., 2003). In contrast, monkeys with selective lesions of the hippocampus performed equally to controls on these tasks (Saksida et al., 2006). This demonstrates that perirhinal lesions impair the discrimination of the conjunction of different features of an object. Similar results were obtained in rats using an ORT with feature ambiguous objects (Bartko et al., 2007). Thus, perceptualization is subserved by the perirhinal cortex and allows this region to similarly signal the novelty of an object.

Using c-fos immunostaining, brain regions activated in animals in response to visual stimulation can be determined. This method involves measuring the amount of c-fos proteins in a certain brain region with immunohistochemistry. The c-fos protein is produced by the c-fos proto-oncogene, which is rapidly activated by neurotransmitters and growth factors during neural activation (Sagar et al., 1988). When familiar and novel stimuli were presented to different sides of the visual field of the rat, novel objects cause an increased activation of the perirhinal cortex and ventral visual stream region TE, but not the hippocampus, compared to familiar objects. In contrast, when familiar objects were put in varying novel spatial arrangements, thereby creating different scenes, the postrhinal cortex and subregion CA1 of the hippocampus showed increased c-fos activation, whereas other hippocampal subregions like the DG and subiculum were significantly deactivated (Wan et al., 1999). This suggests a role for the postrhinal cortex and hippocampus in spatial and scene processing and a potential dissociation within hippocampal subregions. The postrhinal

cortex seems to process the spatial locations, while the hippocampus integrates objects into this spatial context.

Another recent c-fos study similarly demonstrated an activation-increment in especially the caudal part of the perirhinal cortex when novel versus familiar objects were presented to rats in a spontaneous exploration paradigm (Albasser et al., 2010). Similar to the earlier study, no *overall* pattern in c-fos activation was found in the hippocampus: the CA1 and CA3-regions showed a similar pattern to the perirhinal cortex with increased activation in response to novel stimuli, in contrast with the DG responding with a decreased activation to novelty. To investigate this novelty/familiarity dissociation further, structural equation modeling of the variance in c-fos activations revealed two dissociated pathways. One parahippocampal-hippocampal pathway was found, which was formed by the temporo-ammonic pathway (TE2 → perirhinal cortex → entorhinal cortex → and CA1) upon presentation of familiar stimuli. When the presented stimuli were novel, another pathway was found along the perforant pathway (TE2 → perirhinal cortex → entorhinal cortex → DG → CA3 → CA1). Based on these findings, it was argued that the entorhinal cortex might be a switch that can modulate hippocampal processing based on novelty or familiarity of a stimulus (Albasser et al., 2010), in line with the rhinal gateway hypothesis (Fernández and Tendolkar, 2006). The dissociation amongst hippocampal subregions might be explained by considering the proposed functional specialization of hippocampal subregions. These results highlight the intricate dynamics of the interaction between the rhinal cortex and hippocampus, and these results suggest how both structures might contribute to familiarity, recollection and novelty of an event.

Spatial perception and recognition

Objects can usually be seen in a spatial context, in relation to other objects and backgrounds. Therefore, it is worth considering spatial and scene perception and recognition in more detail. As discussed earlier, the ventral visual pathway is hypothesized to form an extension into the perirhinal cortex. The parahippocampal cortex, is believed to be an extension of a dorsal pathway of visual processing in the brain involved in spatial processing, converging with object information in the hippocampus (Eichenbaum, 1997; Mishkin et al., 1997; Kahn et al., 2008; Kravitz et al., 2011). It is interconnected with regions of this dorsal stream, such as the posterior parietal cortex, posterior cingulate cortex and retrosplenial cortex (Kahn et al., 2008; Kravitz et al., 2011). The posterior parietal cortex and posterior cingulate cortex are believed to be involved in spatial attention, preparing actions towards target locations and the egocentric processing of spatial surroundings (Calton and Taube, 2009; Lindner et al., 2010;

Kravitz et al., 2011). The retrosplenial cortex is believed to play an essential role in spatial navigation (Maguire, 2001). Behaviorally, it has been shown that patients with lesions to the right parahippocampal cortex are impaired in various visuospatial memory tasks (Bohbot et al., 1998).

The importance of the parahippocampal cortex in spatial and action-oriented processing was tested in animal models, such as monkeys performing an oculomotor delayed response task (Ploner et al., 2000). Monkeys with lesions of the perirhinal cortex and parahippocampal cortex performed significantly worse than monkeys with lesions to the perirhinal cortex alone or control subjects, underscoring the importance of the parahippocampal cortex in location recognition and location-oriented action. A recent study demonstrated that monkeys with lesions to the perirhinal cortex, parahippocampal cortex or the hippocampal formation performed differently on several visual tasks involving object-in-place associations or spatial locations (Bachevalier and Nemanic, 2008). In the spatial location task, monkeys are made to detect a novel location of one of two familiar objects. In the object-in-place task, a comparison must be made between two images to detect a novel arrangement of five familiar objects. In addition, a classical ORT served as a control task. Lesions of the perirhinal cortex caused a deficit in the object-in-place task and the ORT compared to controls, corroborating this region's importance in object recognition. Lesions of the parahippocampal cortex caused impairments in the object-in-place and spatial location task, but not in the ORT, pointing out the importance of this area in processing the spatial features. Performance in the spatial-location task was uniquely impaired by parahippocampal cortex lesions. Hippocampal lesions also led to impairments on the object-in-place task. These monkey experiments corroborate the importance of the parahippocampal cortex in processing spatial features.

The effect of perirhinal and postrhinal cortex lesions in rats on the performance of object or contextual memory, was tested using the ORT (Norman and Eacott, 2005). Rats explore familiar objects in incongruent but familiar contexts more than objects that appear in congruent contexts, revealing memory for the context in which objects were previously presented. However, lesions of the postrhinal cortex, the rat analogue of the human parahippocampal cortex, caused severe impairments on this task, compared to controls and rats with perirhinal cortex lesions. In contrast, perirhinal cortex lesions caused severe impairments in the ORT compared to controls and postrhinal cortex lesions. Apart from the well-replicated finding that the perirhinal cortex is important for object recognition (e.g. Kim et al., 2014), these findings indicate that the postrhinal cortex plays a role in contextual, spatial processing of scenes (Winters et al., 2008).

Temporal perception and recognition

Thus far, the structures related to recognizing the 'what' and 'where' have been described. However, for an episodic memory a 'when' component is also required (Tulving, 1972). Although relatively few studies have been dedicated to the temporal aspect of episodic memory, the presence of a 'when'-pathway has been suggested in the representation of the moment-to-moment visual world, in the temporal context in the order of seconds. Information gathered from parietal-lobe lesioned patients and 'virtual lesions' induced by transcranial magnetic stimulation studies suggest that this pathway is dedicated to using time information to identify objects based on when objects appeared and disappeared, and whether they appeared successively or simultaneously. The temporoparietal junction was identified as a core anatomical locus within the right inferior parietal lobe; however, the 'when'-pathway is likely to include a bigger network of areas, originating in V1 and including the right angular gyrus, the supramarginal gyrus and the superior temporal gyrus (Battelli et al., 2007). Furthermore, it was shown that the 'when'-pathway is distinct from, and interacts with, the established 'where'- and 'what'-pathways. In addition, it was proposed that each sensory system has its own 'when'-pathway originating in its primary sensory cortex and taking a route through the parietal and related motor cortices for programming responses to the world (Battelli et al., 2008).

To separate the anatomical underpinnings of the object, spatial and temporal elements of episodic memory, Ekstrom and colleagues used a taxi-driver game incorporating retrieval trials that are dissociable in terms of their object, spatial and temporal memory requirements (Ekstrom and Bookheimer, 2007). In this task participants freely searched for passengers to deliver to several landmark stores in a virtual reality environment. In the scanner, they were cued to retrieve landmarks (objects), spatial associations or temporal order from their earlier navigational experience. Both the hippocampus and parahippocampal cortex were activated during all three types of retrieval tasks. The perirhinal cortex was preferentially activated during the landmark retrieval task, in line with predictions. A dissociation was found between the parahippocampal cortex and the hippocampus, where the hippocampus was activated more than the parahippocampal cortex in temporal-retrieval tasks, and the parahippocampal cortex was activated more than the hippocampus in the spatial retrieval task (Ekstrom and Bookheimer, 2007). A follow-up study using the same paradigm revealed that hippocampal activity was not differentiated between temporal and spatial retrieval tasks, whereas the parahippocampal cortex was activated the most during the spatial retrieval task. Interestingly, prefrontal activation was the highest during temporal order retrieval. This is in line with the importance of the hippocampus in representing episodes in the context of space *and* time and the parahippocampal cortex in preferentially representing spatial layout, but

suggests a prefrontal contribution to memory in terms of temporal order (Ekstrom et al., 2011). In humans, fMRI-results show that the orbitofrontal cortex is specifically involved in the encoding and retrieval of temporal order associations, but not spatial associations amongst objects. Similarly, patients with lesions of the orbitofrontal cortex experience impairments in temporal but not spatial source judgment accuracy (Duarte et al., 2009).

Temporal order memory was also studied in rats and mice. Compared to primates the PFC is less differentiated, and the translation of specific anatomical subregions of the PFC is therefore somewhat problematic (Uylings et al., 2003). However, general conclusions can be made about the involvement of prefrontal regions. In rats, temporal recognition memory performance on a temporal order ORT was found to be dependent on an interaction between the hippocampus and either the MPFC or perirhinal cortex (Barker et al., 2007; Warburton and Brown, 2010; Barker and Warburton, 2011). Similarly, in mice the hippocampus and MPFC were essential for temporal order memory, as demonstrated in lesioned mice. The tendency of mice to preferentially explore odors that were presented longer ago disappeared in MPFC-lesioned mice (DeVito and Eichenbaum, 2011). The mouse or rat hippocampus appears to integrate 'what' information with 'where' and 'when' information, as lesioning the hippocampus impairs performance of all three aspects in an ORT-task (Li and Chao, 2008; DeVito and Eichenbaum, 2010).

To investigate how item and timing information is integrated in the MTL in nonhuman primates, neuronal activity was recorded while they performed a temporal-order memory task requiring the encoding of two visual items and their temporal order. It was found that the perirhinal cortex appears to integrate timing information from the hippocampus, via the entorhinal cortex with item information from the visual sensory area TE (Naya and Suzuki, 2011). These studies might reveal how temporal processing is performed over short intervals (spanning milliseconds to seconds), to enable re-experiencing short but fluid segments of events. However, the coding of the temporal element of episodic memory remains quite speculative. Based on these results, it can preliminarily be concluded that the hippocampus, PFC and perirhinal cortex are important for the representation of temporal order aspects in episodic memory (Wilson et al., 2010). More studies will be needed to elucidate how this potential 'when'-pathway functions in relation to episodic memory and the functioning of the MTL.

The prefrontal cortex and recognition memory

We have come across the PFC in the context of temporal order memory. Animal models using a crossed unilateral asymmetrical ablation method generally suggest the importance of an intact connection between the PFC and the hippocampus in episodic memory. This method involves the crossed unilateral asymmetrical ablation of each of two interconnected brain regions. This allows for assessing the influence of an absence of a connection amongst two intact regions, on top of the contribution of the respective brain regions themselves. The alternative method is to cut specific connecting axons, but this is very difficult to achieve without damaging surrounding neurons. Studies using a crossed unilateral lesioning-paradigm show that the connections between an intact inferior temporal cortex and PFC in monkeys are for example required for the maintenance of a visual learning set (Browning et al., 2007), and object-in-place learning (Browning et al., 2005; Wilson et al., 2008). Selective ablation studies in monkeys and rats show a differential involvement in recognition memory tasks of the perirhinal cortex, hippocampus and the PFC. For the ORT, an intact perirhinal cortex is required, whereas for an object location task, to measure spatial memory, performance depends on an intact hippocampus. In contrast, when object and location need to be combined in the object-in-place task, or when the task is to judge which of two objects has been presented longer ago (temporal order task), the perirhinal cortex, hippocampus and the MPFC are all required for normal performance. Performance of a temporal-order and the object-in-place task thus depends on an intact circuitry amongst these three regions (Warburton and Brown, 2010). This alludes to a general involvement of the PFC in recollective episodic memory. Similarly, studies of rats with bilaterally lesioned MPFC showed an impaired recollective, but not familiarity-performance on DNMS-tasks that measure single item/object recognition (Farovik et al., 2008). Even though findings of prefrontal functioning are difficult to generalize across species, these animal models suggest that the PFC is essential for recollection.

To explain the potential role of the human PFC in memory and recollection, it can be argued that declarative memory relies on two interacting components (Moscovitch, 2008; Shing et al., 2008). The hippocampal component is associative, and consists of the mechanisms by which different features of the memory content are combined into a coherent representation or trace. The hippocampal output guides semantic, implicit and perceptual processes, without necessarily inducing a conscious appraisal of its contents. The second strategic prefrontal component is a slower, higher-level process with recollective output that can be explicitly and consciously manipulated to guide behavior (Moscovitch, 2008). It consists of control processes that regulate

memory encoding and retrieval, allowing for both the elaboration and organization of the memory content, as well as monitoring and evaluating the retrieved information (Blumenfeld and Ranganath, 2006; Badre and Wagner, 2007; Blumenfeld and Ranganath, 2007; Shing et al., 2008; Blumenfeld et al., 2011).

Automatic associative recognition memory can occur independently of top-down control, and might not require activation of frontal regions (Simons and Spiers, 2003). However, prefrontal processes are especially needed for free recall, as the absence of external cues requires strategic memory processes to guide internal cueing of retrieval. Free recall requires more internal elaboration and organization of memory than associative recognition. The PFC is needed for free recall to allow top-down conscious control of retrieval, ensuring that a coherent representation is retrieved from the MTL and made available for working memory.

Human fMRI-evidence indicates that the DLPFC is activated uniquely in free recall, but not in associative recognition (Staresina and Davachi, 2006). Interestingly, DLPFC-activity during the encoding of words has been found to be related to the production of a cluster of semantically related words during free recall. A method of semantic clustering might be one of the organizational processes that potentially aids free recall (Long et al., 2010). Also, placing immediate recollective experiences in the wider context of a source would aid retrieval of temporal and spatial source. The DLPFC thus appears to be important for the elaboration and organization of memory traces in working memory (Blumenfeld and Ranganath, 2006). The DLPFC also has the role of forming and strengthening associations amongst certain items, events, or scenes represented by MTL-areas, and of associations between the semantic nodes in a neocortical schema or knowledge structure (Fletcher and Henson, 2001; Blumenfeld and Ranganath, 2006; Cruse and Wilding, 2009; Hayama and Rugg, 2009; Long et al., 2010). The involvement of DLPFC in post-retrieval judgments was found to be equal whether participants were required to make source (episodic) judgments about pictures or they made judgments about semantic attributes (Hayama and Rugg, 2009). This suggests the DLPFC is important for elaborative processing of both episodic and semantic memory.

DLPFC-activity was also found to be specifically involved in elaborative encoding tasks where participants were cued to process the relations of items, whereas the VLPFC was activated equally in elaborative and nonelaborative encoding (Blumenfeld et al., 2011). The human VLPFC in turn is found to be important for the selection of goal-relevant items during encoding and retrieval, thereby strengthening representations of goal-relevant features (Fletcher and Henson,

2001; Blumenfeld and Ranganath, 2007; Mitchell and Johnson, 2009). It controls the conscious access to stored conceptual semantic information, as well as performing post-retrieval monitoring of active representations to resolve competition (Badre and Wagner, 2007).

Information that is congruent with existing knowledge structures are easily embedded in a schematic network. Indeed, similar results were found in rats. In paired-associate tasks, rats learn the associations of flavors with places in an event arena. This task requires the learning of persistent flavor-place associations, which is dependent on hippocampal mediation. However, as a neocortical schema of the associations developed over time, new flavor-place associations were, already after one presentation, assimilated in the neocortical schema. In fact, rats with a lesioned hippocampus were able to acquire new flavor-place associations within one trial, as well as subsequently retrieve these paired associations (Tse et al., 2007). Whereas the MTL is responsible for laying down our experiences implicitly in recollective representations, the MPFC seems to assimilate information from these experiences on the basis of earlier experiences, existing schemas and goals. It is then able to explicitly and consciously manipulate this information by elaborating and organizing the contents of semantic memory during encoding as well as monitoring and evaluating this information during retrieval.

Towards a unifying neuropsychobiological framework of *recognition and recall*

What / Where / When

Substantial progress has been made in delineating the function of several structures and subsystems involved in processing incoming visual information. The visual system has been subdivided into over thirty functionally distinct areas and two visual streams, a dorsal ‘where’-pathway and a ventral ‘what’-pathway, that project from the primary visual cortex to higher association areas (Ungerleider and Haxby, 1994). The ventral pathway is hypothesized to be concerned with processing mainly the specific features of an object, i.e., ‘what’ the object is. The dorsal pathway on the other hand, is concerned with processing the context of an object, i.e., ‘where’ an object is located in visual space and the spatial arrangement of scenes. From studies on object and spatial recognition it has become clear that the perirhinal cortex is important in particularly visual object recognition and perception, and the parahippocampal cortex plays a crucial role in particularly spatial recognition and perception. Studies on the anatomical connections of the perirhinal and parahippocampal cortex suggest they form a possible extension of the ‘what’ and ‘where’-streams

(Eichenbaum et al., 2007). The older conceptualization of the 'what' and 'where' visual streams has been challenged by a newer conceptualization emphasizing the function of the two streams, namely 'dorsal' vision for action, and 'ventral' vision for perception (Milner and Goodale, 2008). However, we are mainly interested in the implications for the functioning of the MTL, the main one being that the visual input of the rhinal regions to the hippocampus might be dissociable along the object/spatial context distinction. Future studies should aim to find a double dissociation between the involvement of perirhinal cortex and parahippocampal cortex in respectively vision for perception and vision for action. Temporal information needs to be processed to form a recollection, and it is processed by regions such as the perirhinal cortex and PFC together with the hippocampus. A 'when'-pathway might converge in the hippocampus (e.g. Naya and Suzuki, 2011), but compelling evidence on this hypothesis is presently lacking.

Familiarity vs. Recollection

Modality-specific processing in cortical areas generally show a convergence of information towards higher-order association areas (Mishkin et al., 1997). In the visual system, higher-order areas represent increasingly bigger receptive fields and an increasing amount of different features of a stimulus (e.g. color, brightness, shape, and texture). This hierarchical convergence can be extended to the perirhinal cortex, where object features converge from the ventral visual stream to allow the unitized processing of complex configural stimuli; and the parahippocampal cortex, where spatial information converges from the dorsal visual stream into one scene/arrangement representation. Object and spatial representations in these regions allow for the perception, as well as recognition of specific exemplars. This information is transferred through the lateral and medial entorhinal cortex, respectively, to the final point of convergence in the hippocampus. The hippocampus can be assumed to be the unique point of convergence of polymodal stimulus and spatiotemporal context information. The hippocampus combines the information that allows reliving and remembering an event with all its contextual details.

Electrophysiological, pharmacological and lesion studies in rodents and monkeys, as well as human brain imaging and behavioral performance measures, suggest that familiarity and recollection can be dissociated. The perirhinal cortex represents object information, whereas the parahippocampal cortex represents spatial/scene information, and based upon a match/mismatch signal of the incoming representations and stored representations, they signal the novelty or familiarity of the object. In our view, this also applies to the familiarity of context. Incoming information from the perirhinal and

parahippocampal cortex is combined in the entorhinal cortex which probes the hippocampus to either encode or retrieve an episodic trace. This might be accomplished through a familiarity/novelty switch in the entorhinal cortex, activating two potentially distinct pathways through the hippocampus (Fernández and Tendolkar, 2006; Albasser et al., 2010). This allows for the allocation of limited encoding/storage resources of experiential episodic traces towards relevant and often novel information. When information of a familiar element is present in the rhinal cortex, the episodic trace can be completed in the hippocampus through a different pathway. It seems reasonable to postulate some form of Dual-Process Theory, even though the dissociation of familiarity and recollection might only go in one direction: familiarity can exist without recollection, but recollection could depend on familiarity-signals and individual episodic elements (Zola-Morgan et al., 1989).

MTL vs. PFC

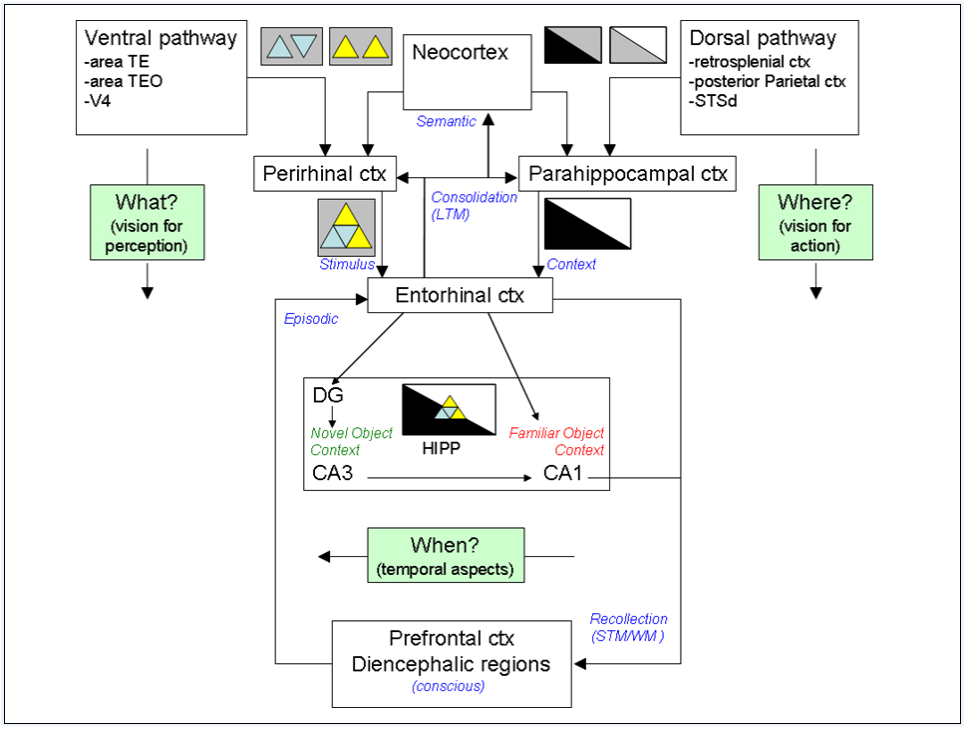
The function of the PFC is to control and regulate memory encoding and retrieval, allowing for both the elaboration and organization of the memory content, as well as monitoring and evaluating the retrieved information. It therefore guides conscious access to, and manipulation of, information in episodic and semantic memory. The MTL is an automatic learning system which stores unique episodic experiences, and over time the slower-learning knowledge system in the neocortex can extract relevant information from episodic representations. The PFC enables the extraction of statistical regularities and relations from episodic information by embedding them in a schema or knowledge structure. As such, the gist, or semantic core is extracted to allow the most important information to be preserved from episodic experiences. All except the most meaningful representations of experiences lose episodic detail over time, thus becoming less vivid. The schema also appears to be a catalyst for abstraction of new episodic information into a semantic schema, based on interactions between MTL and PFC. Even though the PFC oversees this ‘semanticization’, the semantic knowledge itself could be stored elsewhere in the neocortex (Patterson et al., 2007). In this sense, interactions of the MTL with the PFC are important for extracting regularities by comparing input from experience with predicted information to extract regularities (Henson and Gagnepain, 2010). The role of this interplay to extract regularities and relations can be contrasted with the role of the MTL itself in detecting novelty on the basis of predictions, to determine which episodic traces to store (Fernández and Tendolkar, 2006).

Therefore, incoming information from experiences is filtered on two levels. At the level of the rhinal cortex, novelty of an experience determines what detailed

episodic memories are encoded, and which ones quickly fade. It makes sense to store especially novel relevant experiences vividly, instead of storing every event or human experience that occurs on a day with the same sensory detail. The second level of filtering determines what information is stored in robust semantic mental schemas, on the basis of rules, regularity and predictability ('accidents often happen on icy roads'). Novel memories allow for useful predictions about the future ('I should not drive too fast here, as an accident occurred here earlier') as well as regularities ('the road is icy, so I better not drive fast'). According to this two-stage process, novel experiences provide information for later semantic learning, but familiar experiences can also lead to direct semantic learning if its contents are congruent with an existing scheme. Moreover, the PFC monitors and retrieves information in episodic and semantic representations and manipulates information on the basis of goal-relevant motivations. The rhinal cortex bases its novelty-judgment on predictions from existing sensory, episodic and semantic representations, whereas the MPFC extracts regularities and regulations on the basis of predictions from the existing schema and experiences. Therefore incoming experiences in episodic and semantic memory serve to make predictions about future experiences more accurate, and this serves to better guide our behavior in interacting with the environment (Henson and Gagnepain, 2010).

Unifying neuropsychobiological framework

Memory systems can be independent, synergistic or competitive (Kim and Baxter, 2001). We regard memory systems as synergistic. Accordingly, recollection complements familiarity, by means of the episodic system adding contextual information to a familiar feeling of knowing an object (Eichenbaum et al., 2007) and similarly, episodic and semantic information influence each other. In Figure 2, our novel unified framework of recognition and recall memory is presented.



Ctx = cortex, V4 = Visual area 4, TE = cytoarchitectonic area in inferior temporal lobe, TEO = cytoarchitectonic area in inferior temporal lobe, STSd = dorsal Superior Temporal Sulcus, DG = Dentate Gyrus, CA1-CA3 = Cornu Ammonis regions 1 and 3, Hipp = Hippocampus, WM = Working Memory, LTM = Long-Term Memory, STM = Short-Term Memory.

Figure 2. A schematic overview of our proposed framework of the episodic memory system. Brain structures of the dorsal and ventral visual streams containing respectively object and spatial/context information, converge into the hippocampus. The representation of visual features in these brain regions subserves both perception and recognition. Temporal information is processed in the inferior parietal lobe, and through the perirhinal cortex and prefrontal cortex integrated in the hippocampus. The hippocampus relates a familiar object, odor, sound or an internal thought of a stimulus with its spatiotemporal context to form one complete episodic memory. Alternatively, upon the detection of a novel episodic element, new recollective representations are formed by the hippocampus. For complete conscious recollection, i.e. recognition in case of a familiar stimulus or recall in case of an internal thought of a stimulus, a representation needs access to working memory in the prefrontal cortex. The prefrontal cortex controls and regulates memory encoding and retrieval, allowing for both the elaboration and organization of the memory content, as well as monitoring and evaluating the retrieved information. Subsequently, both the old as well as newly-formed explicit/declarative memory traces (in case of a novel stimulus or context) can then be reconsolidated and consolidated, respectively. The consolidation takes place in the perirhinal and parahippocampal cortices or as semanticized memory traces in the neocortex. Finally, these memory traces can be retrieved again in subsequent recollections to dynamically form episodic memories.

This framework is based on the assumptions mentioned above, and incorporates the most recent findings on connections between different relevant brain regions and the information processing functions of these regions. For a comprehensive explanation see legend of Figure 2.

Conclusion

Many reviews have appeared that consider the complex episodic memory system in the brain (e.g. Eichenbaum et al., 2007; Squire et al., 2007; Bird and Burgess, 2008; Dickerson and Eichenbaum, 2009; Wang and Morris, 2010; Nadel and Hardt, 2011; Wixted and Squire, 2011b). This indicates that the subject is both fundamental and topical. We synthesize contemporary ideas about brain regions and their connections in episodic memory functioning, as well as the information represented in brain regions. Our comprehensive model synthesizes seemingly contradictory processes and findings such as: the encoding of novel and schema-congruent information, conscious and unconscious memory processes, familiarity and recollection, semantic and episodic memory, perception and memory. The model encompasses the intricate and dynamic interaction of several brain regions (PFC, perceptual association areas and the MTL) that enable an accurate and useful representation of our experiences for future references. Representations are formed, modified and also degraded in response to perceiving new information and changing internal motivational processes. They constantly change in response to new experiences, and serve to increase the accuracy of our prediction of future experiences.

Taking everything into consideration, we hypothesize that the presence of a familiar object, odor, sound, but also context or the spontaneous thought of a stimulus or context, leads to an episodic recollection in the hippocampus by connecting stimulus and contextual information from different extrahippocampal areas. For complete conscious recollection, a representation has to be transferred from the hippocampal formation to the PFC into working memory. Subsequently, both this old as well as a new episodic memory trace, in case of a novel stimulus or context, can then be reconsolidated and consolidated, respectively, into the extrahippocampal areas as an episodic memory, or be semanticized into the neocortex as abstract representations. These representations can subsequently be retrieved again in subsequent recollections. These assumptions need to be investigated in future studies which thoroughly dissect the different parts and processes of declarative memory and determine how specific brain areas and signaling pathways contribute to recognition memory.

Our model leads to new predictions on specific brain substrates for familiarity, a separate modality for familiarity of context, and that temporal information is labelled to a certain stimulus (object or context) information. To test these predictions we need higher resolution imaging, precise lesion studies in animals, and subcortical electrophysiology (TMS, EEG). For instance, multivariate pattern analysis techniques of fMRI-data allow us to probe the representational content of brain regions (Norman et al., 2006; Hassabis et al., 2009; Chadwick et al., 2010; Bonnici et al., 2011; Kriegeskorte 2011). New analysis techniques allow us to assess the nature of functional connectivity between brain regions (Poppenk et al., 2010; van Kesteren et al., 2010; Schott et al., 2011). Using high-resolution imaging, we might be able to dissociate brain regions that have been often regarded as one functional unit, for example the subregions of the hippocampus (Thomas et al., 2008; Ekstrom et al., 2009; Carr et al., 2010). Similarly, animal and human studies should focus on developing innovative translational behavioral paradigms to search for dissociations of processes and brain regions. For instance, electrophysiological measurements can probe the nature of the time course of both encoding and retrieval more directly in both humans and animals, elucidating the role of gamma- and theta-waves, spiking patterns and neurotransmitter levels. We hope that our present model is useful in guiding future research efforts into the intricate mechanisms by which functional integration allows the brain to retain information from our experiences for future reference.

References

Aggleton, J. P., and Brown, M. W. (1999). Episodic memory, amnesia, and the hippocampal-anterior thalamic axis. *Behavioral and Brain Sciences*, *22*(3), 425-489.

Aggleton, J. P., and Brown, M. W. (2006). Interleaving brain systems for episodic and recognition memory. *Trends in Cognitive Sciences, 10*(10), 455-463.

Agster, K. L., and Burwell, R. D. (2009). Cortical efferents of the perirhinal, postrhinal, and entorhinal cortices of the rat. *Hippocampus, 19*(12), 1159-1186.

Akkerman, S., Blokland, A., Reneerkens, O., van Goethem, N. P., Bollen, E., Gijselaers, H. J. M., Lieben, C. K. J., Steinbusch, H. W. M., and Prickaerts, J. (2012). Object recognition testing: Methodological considerations on exploration and discrimination measures. *Behavioural Brain Research*, *232*(2), 335-347.

Albasser, M. M., Davies, M., Futter, J. E., and Aggleton, J. P. (2009). Magnitude of the object recognition deficit associated with perirhinal cortex damage in rats: Effects of varying the lesion extent and the duration of the sample period. *Behavioral Neuroscience*, *123*(1), 115-124.

Albasser, M. M., Poirier, G. L., and Aggleton, J. P. (2010). Qualitatively different modes of perirhinal–hippocampal engagement when rats explore novel vs. familiar objects as revealed by c Fos imaging. *European Journal of Neuroscience, 31*(1), 134-147.

Andersen, P. (2007). The hippocampus book: Oxford University Press.

Bachevalier, J., and Nemanic, S. (2008). Memory for spatial location and object place associations are differently processed by the hippocampal formation, parahippocampal areas TH/TF and perirhinal cortex. Hippocampus, *18*(1), 64-80.

Badre, D., and Wagner, A. D. (2007). Left ventrolateral prefrontal cortex and the cognitive control of memory. *Neuropsychologia, 45*(13), 2883-2901.

Barbas, H., and Blatt, G. J. (1995). Topographically specific hippocampal projections target functionally distinct prefrontal areas in the rhesus monkey. *Hippocampus, 5*(6), 511-533.

Barker, G. R. I., Bird, F., Alexander, V., and Warburton, E. C. (2007). Recognition Memory for Objects, Place, and Temporal Order: A Disconnection Analysis of the Role of the Medial Prefrontal Cortex and Perirhinal Cortex. *The Journal of Neuroscience*, *27*(11), 2948-2957.

Barker, G. R. I., and Warburton, E. C. (2011). When Is the Hippocampus Involved in Recognition Memory? *The Journal of Neuroscience*, *31*(29), 10721-10731.

Bartko, S. J., Winters, B. D., Cowell, R. A., Saksida, L. M., and Bussey, T. J. (2007). Perceptual functions of perirhinal cortex in rats: Zero-delay object recognition and simultaneous oddity discriminations. *The Journal of Neuroscience, 27*(10), 2548-2559.

Battelli, L., Pascual-Leone, A., and Cavanagh, P. (2007). The 'when' pathway of the right parietal lobe. *Trends in Cognitive Sciences*, *11*(5), 204-210.

Battelli, L., Walsh, V., Pascual-Leone, A., and Cavanagh, P. (2008). The 'when' parietal pathway explored by lesion studies. *Current Opinion in Neurobiology, 18*(2), 120-126.

Bayley, P. J., O'Reilly, R. C., Curran, T., and Squire, L. R. (2008). New semantic learning in patients with large medial temporal lobe lesions. *Hippocampus, 18*(6), 575-583.

Bird, C. M., and Burgess, N. (2008). The hippocampus and memory: insights from spatial processing. *Nature Reviews Neuroscience*, *9*(3), 182-194.

Blumenfeld, R. S., Parks, C. M., Yonelinas, A. P., and Ranganath, C. (2011). Putting the Pieces Together: The Role of Dorsolateral Prefrontal Cortex in Relational Memory Encoding. *Journal of Cognitive Neuroscience*, *23*(1), 257-265.

Blumenfeld, R. S., and Ranganath, C. (2006). Dorsolateral prefrontal cortex promotes long-term memory formation through its role in working memory organization. *The Journal of Neuroscience*, *26*(3), 916-925.

Blumenfeld, R. S., and Ranganath, C. (2007). Prefrontal Cortex and Long-Term Memory Encoding: An Integrative Review of Findings from Neuropsychology and Neuroimaging. *The Neuroscientist*, *13*(3), 280-291.

Bohbot, V. D., Kalina, M., Stepankova, K., Spackova, N., Petrides, M., and Nadel, L. (1998). Spatial memory deficits in patients with lesions to the right hippocampus and to the right parahippocampal cortex. *Neuropsychologia, 36*(11), 1217-1238.

Bonnici, H. M., Kumaran, D., Chadwick, M. J., Weiskopf, N., Hassabis, D., and Maguire, E. A. (2012). Decoding representations of scenes in the medial temporal lobes. *Hippocampus*, *22*(5), 1143-1153.

Bowles, B., Crupi, C., Mirsattari, S. M., Pigott, S. E., Parrent, A. G., Pruessner, J. C., et al. (2007). Impaired familiarity with preserved recollection after anterior temporal-lobe resection that spares the hippocampus. *Proceedings of the National Academy of Sciences, 104*(41), 16382-16387.

Browning, P. G. F., Easton, A., Buckley, M. J., and Gaffan, D. (2005). The role of prefrontal cortex in object in place learning in monkeys. *European Journal of Neuroscience, 22*(12), 3281-3291.

Browning, P. G. F., Easton, A., and Gaffan, D. (2007). Frontal–temporal disconnection abolishes object discrimination learning set in macaque monkeys. *Cerebral Cortex*, *17*(4), 859-864.

Buckley, M. J., and Gaffan, D. (1998). Perirhinal cortex ablation impairs visual object identification. *The Journal of Neuroscience*, *18*(6), 2268-2275.

Buffalo, E. A., Bellgowan, P. S. F., and Martin, A. (2006). Distinct roles for medial temporal lobe structures in memory for objects and their locations. *Learning and Memory*, *13*(5), 638-643.

Burgess, N., Maguire, E. A., and O'Keefe, J. (2002). The Human Hippocampus and Spatial and Episodic Memory. *Neuron, 35*(4), 625-641.

Burwell, R. D. (2000). The parahippocampal region: corticocortical connectivity. *Annals of the New York Academy of Sciences*, *911*(1), 25-42.

Bussey, T. J., and Saksida, L. M. (2002). The organization of visual object representations: a connectionist model of effects of lesions in perirhinal cortex. *European Journal of Neuroscience*, *15*(2), 355-364.

Bussey, T. J., Saksida, L. M., and Murray, E. A. (2003). Impairments in visual discrimination after perirhinal cortex lesions: testing'declarative'vs. 'perceptual-mnemonic'views of perirhinal cortex function. *European Journal of Neuroscience*, *17*(3), 649-660.

Cabeza, R., and St. Jacques, P. (2007). Functional neuroimaging of autobiographical memory. *Trends in Cognitive Sciences*, *11*(5), 219-227.

Calton, J. L., and Taube, J. S. (2009). Where am I and how will I get there from here? A role for posterior parietal cortex in the integration of spatial information and route planning. *Neurobiology of Learning and Memory, 91*(2), 186-196.

Carr, V. A., Rissman, J., and Wagner, A. D. (2010). Imaging the Human Medial Temporal Lobe with High-Resolution fMRI. *Neuron, 65*(3), 298-308.

Chadwick, M. J., Hassabis, D., Weiskopf, N., and Maguire, E. A. (2010). Decoding Individual Episodic Memory Traces in the Human Hippocampus. *Current Biology*, *20*(6), 544-547.

Cohen, N., and Squire, L. (1980). Preserved learning and retention of pattern-analyzing skill in amnesia: dissociation of knowing how and knowing that. *Science*, *210*(4466), 207-210.

Cohen, N. J., and Eichenbaum, R. A. P. H. (1997). Memory for Items and Memory for Relations in the Procedural/Declarative Memory Framework. *Memory*, *5*(1-2), 131-178.

Corkin, S. (1968). Acquisition of motor skill after bilateral medial temporal-lobe excision. *Neuropsychologia*, *6*(3), 255-265.

Corkin, S. (2002). What's new with the amnesic patient HM? *Nature Reviews Neuroscience, 3*(2), 153-160.

Cowell, R. A., Bussey, T. J., and Saksida, L. M. (2006). Why does brain damage impair memory? A connectionist model of object recognition memory in perirhinal cortex. *The Journal of Neuroscience*, *26*(47), 12186-12197.

Cruse, D., and Wilding, E. L. (2009). Prefrontal cortex contributions to episodic retrieval monitoring and evaluation. *Neuropsychologia, 47*(13), 2779-2789.

Daselaar, S. M., Fleck, M. S., and Cabeza, R. (2006). Triple dissociation in the medial temporal lobes: Recollection, familiarity, and novelty. *Journal of Neurophysiology*, *96*(4), 1902-1911.

Davachi, L. (2006). Item, context and relational episodic encoding in humans. *Current Opinion in Neurobiology*, *16*(6), 693-700.

DeVito, L. M., and Eichenbaum, H. (2010). Distinct contributions of the hippocampus and medial prefrontal cortex to the "what–where–when" components of episodic-like memory in mice. *Behavioural Brain Research, 215*(2), 318-325.

DeVito, L. M., and Eichenbaum, H. (2011). Memory for the Order of Events in Specific Sequences: Contributions of the Hippocampus and Medial Prefrontal Cortex. *The Journal of Neuroscience*, *31*(9), 3169-3175.

Diana, R., and Ranganath, C. (2011). Recollection, familiarity and memory strength: confusion about confounds. *Trends in Cognitive Sciences*, *15*(8), 337-338.

Diana, R. A., Yonelinas, A. P., and Ranganath, C. (2007). Imaging recollection and familiarity in the medial temporal lobe: a three-component model. *Trends in Cognitive Sciences, 11*(9), 379-386.

Diana, R. A., Yonelinas, A. P., and Ranganath, C. (2008). The effects of unitization on familiarity-based source memory: Testing a behavioral prediction derived from neuroimaging data. *Journal of Experimental Psychology: Learning, Memory, and Cognition, 34*(4), 730-740.

Dickerson, B. C., and Eichenbaum, H. (2009). The episodic memory system: neurocircuitry and disorders. *Neuropsychopharmacology, 35*(1), 86-104.

Duarte A., Henson R., Knight R., Emery T., Graham K. (2010). Orbito-frontal Cortex is Necessary for Temporal Context Memory. *Journal of Cognitive Neuroscience, 22*(8), 1819-1831

Eacott, M. J., Gaffan, D., and Murray, E. A. (1994). Preserved recognition memory for small sets, and impaired stimulus identification for large sets, following rhinal cortex ablations in monkeys. *European Journal of Neuroscience, 6*(9), 1466-1478.

Eichenbaum, H. (1997). How does the brain organize memories? *Science, 277*(5324), 330-332.

Eichenbaum, H. (1999). The hippocampus: The shock of the new. *Current Biology, 9*(13), 482-484.

Eichenbaum, H., Yonelinas, A. R., and Ranganath, C. (2007). The medial temporal lobe and recognition memory. *Annual Review of Neuroscience*, *30*, 123-152.

Ekstrom, A. D., Bazih, A. J., Suthana, N. A., Al-Hakim, R., Ogura, K., Zeineh, M., et al. (2009). Advances in high-resolution imaging and computational unfolding of the human hippocampus. *NeuroImage, 47*(1), 42-49.

Ekstrom, A. D., and Bookheimer, S. Y. (2007). Spatial and temporal episodic memory retrieval recruit dissociable functional networks in the human brain. *Learning and Memory*, *14*(10), 645-654.

Ekstrom, A. D., Copara, M. S., Isham, E. A., Wang, W.-c., and Yonelinas, A. P. (2011). Dissociable networks involved in spatial and temporal order source retrieval. *NeuroImage, 56*(3), 1803-1813.

Eldridge, L. L., Knowlton, B. J., Furmanski, C. S., Bookheimer, S. Y., and Engel, S. A. (2000). Remembering episodes: A selective role for the hippocampus during retrieval. *Nature Neuroscience*, *3*, 1149-1152.

Ennaceur, A. (2010). One-trial object recognition in rats and mice: Methodological and theoretical issues. *Behavioural Brain Research*, *215*(2), 244-254.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*(1), 47-59.

Farovik, A., Dupont, L. M., Arce, M., and Eichenbaum, H. (2008). Medial Prefrontal Cortex Supports Recollection, But Not Familiarity, in the Rat. *Journal of Neuroscience*, *28*(50), 13428-13434.

Fernández, G., and Tendolkar, I. (2006). The rhinal cortex: 'gatekeeper' of the declarative memory system. *Trends in Cognitive Sciences*, *10*(8), 358-362.

Fletcher, P. C., and Henson, R. N. A. (2001). Frontal lobes and human memory. *Brain, 124*(5), 849-881.

Fortin, N. J., Wright, S. P., and Eichenbaum, H. (2004). Recollection-like memory retrieval in rats is dependent on the hippocampus. *Nature, 431*(7005), 188-191.

Frankland, P. W., and Bontempi, B. (2005). The organization of recent and remote memories. *Nature Reviews Neuroscience*, *6*(2), 119-130.

Fuji, T., Moscovitch, M., and Nadel, L. (2000). Memory consolidation, retrograde amnesia, and the temporal lobe (2 ed.). London: Elsevier.

Furtak, S. C., Wei, S. M., Agster, K. L., and Burwell, R. D. (2007). Functional neuroanatomy of the parahippocampal region in the rat: The perirhinal and postrhinal cortices. *Hippocampus, 17*(9), 709-722.

Gilboa, A., Winocur, G., Grady, C. L., Hevenor, S. J., and Moscovitch, M. (2004). Remembering our past: functional neuroanatomy of recollection of recent and very remote personal events. *Cerebral Cortex*, *14*(11), 1214-1225.

Haskins, A. L., Yonelinas, A. P., Quamme, J. R., and Ranganath, C. (2008). Perirhinal cortex supports encoding and familiarity-based recognition of novel associations. *Neuron, 59*(4), 554-560.

Hassabis, D., Chu, C., Rees, G., Weiskopf, N., Molyneux, P. D., and Maguire, E. A. (2009). Decoding Neuronal Ensembles in the Human Hippocampus. *Current Biology*, *19*(7), 546-554.

Hayama, H. R., and Rugg, M. D. (2009). Right dorsolateral prefrontal cortex is engaged during post-retrieval processing of both episodic and semantic information. *Neuropsychologia, 47*(12), 2409-2416.

Henke, K. (2010). A model for memory systems based on processing modes rather than consciousness. *Nature Reviews Neuroscience*, *11*(7), 523-532.

Henson, R. N., and Gagnepain, P. (2010). Predictive, interactive multiple memory systems. *Hippocampus*, *20*(11), 1315-1326.

Hirshhorn, M., Grady, C., Rosenbaum, R. S., Winocur, G., and Moscovitch, M. (2012). The hippocampus is involved in mental navigation for a recently learned, but not a highly familiar environment: A longitudinal fMRI study. *Hippocampus*, *22*(4), 842-852.

Hoover, W., and Vertes, R. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure and Function*, *212*(2), 149-179.

Hoscheidt, S. M., Nadel, L., Payne, J., and Ryan, L. (2010). Hippocampal activation during retrieval of spatial context from episodic and semantic memory. *Behavioural Brain Research*, *212*(2), 121-132.

Hunsaker, M. R., Tran, G. T., and Kesner, R. P. (2008). A double dissociation of subcortical hippocampal efferents for encoding and consolidation/retrieval of spatial information. *Hippocampus, 18*(7), 699-709.

Kahn, I., Andrews-Hanna, J. R., Vincent, J. L., Snyder, A. Z., and Buckner, R. L. (2008). Distinct cortical anatomy linked to subregions of the medial temporal lobe revealed by intrinsic functional connectivity. *Journal of Neurophysiology, 100*(1), 129-139.

Kim, J. J., and Baxter, M. G. (2001). Multiple brain-memory systems: the whole does not equal the sum of its parts. *Trends in Neurosciences*, *24*(6), 324-330.

Kim, J., Delcasso, S., and Lee, I. (2011). Neural correlates of object-in-place learning in hippocampus and prefrontal cortex. *The Journal of Neuroscience, 31*(47), 16991-17006.

Kim, J. M., Kim, D. H., Lee, Y., Park, S. J., and Ryu, J. H. (2014). Distinct roles of the hippocampus and perirhinal cortex in GABAA receptor blockade-induced enhancement of object recognition memory. *Brain Research*, *1552*, 17-25.

Kingsley, R. E., Gable, S. R., and Kingsley, T. R. (1999). Concise text of neuroscience (2 ed.). Baltimore: Lippincott, Williams and Wilkins.

Knowlton, B. J., and Squire, L. R. (1995). Remembering and knowing: two different expressions of declarative memory. *Journal of Experimental Psychology: Learning, Memory, and Cognition, 21*(3), 699-710.

Kravitz, D. J., Saleem, K. S., Baker, C. I., and Mishkin, M. (2011). A new neural framework for visuospatial processing. *Nature Reviews Neuroscience, 12*(4), 217-230.

Kriegeskorte, N. (2011). Pattern-information analysis: From stimulus decoding to computational-model testing. *NeuroImage*, *56*(2), 411-421.

Kumaran, D., and Maguire, E. A. (2005). The human hippocampus: cognitive maps or relational memory? *The Journal of Neuroscience*, *25*(31), 7254-7259.

Lah, S., and Miller, L. (2008). Effects of Temporal Lobe Lesions on Retrograde Memory: A Critical Review. *Neuropsychology Review*, *18*(1), 24-52.

Lavenex, P., and Amaral, D. G. (2000). Hippocampal-neocortical interaction: A hierarchy of associativity. *Hippocampus, 10*(4), 420-430.

LeDoux, J. E. (1993). Emotional Memory: In Search of Systems and Synapses. *Annals of the New York Academy of Sciences*, *702*(1), 149-157.

Li, J. S., and Chao, Y. S. (2008). Electrolytic lesions of dorsal CA3 impair episodic-like memory in rats. *Neurobiology of Learning and Memory, 89*(2), 192-198.

Libby, L. A., Ekstrom, A. D., Ragland, J. D., and Ranganath, C. (2012). Differential connectivity of perirhinal and parahippocampal cortices within human hippocampal subregions revealed by high-resolution functional imaging. *The Journal of Neuroscience*, *32*(19), 6550-6560.

Lindner, A., Iyer, A., Kagan, I., and Andersen, R. A. (2010). Human Posterior Parietal Cortex Plans Where to Reach and What to Avoid. *The Journal of Neuroscience, 30*(35), 11715-11725.

Long, N. M., Öztekin, I., and Badre, D. (2010). Separable Prefrontal Cortex Contributions to Free Recall. *The Journal of Neuroscience*, *30*(33), 10967-10976.

MacLean, P. D. (1955). The limbic system ('visceral brain') and emotional behavior. *AMA Archives of Neurology and Psychiatry*, *73*(2), 130-134.

Maguire, E. (2001). The retrosplenial contribution to human navigation: a review of lesion and neuroimaging findings. *Scandinavian Journal of Psychology*, *42*(3), 225-238.

Maguire, E. A., Frackowiak, R. S. J., and Frith, C. D. (1997). Recalling routes around London: Activation of the right hippocampus in taxi drivers. *The Journal of Neuroscience, 17*(18), 7103-7110.

Mayes, A. R., Montaldi, D., Spencer, T. J., and Roberts, N. (2004). Recalling spatial information as a component of recently and remotely acquired episodic or semantic memories: an fMRI study. *Neuropsychology*, *18*(3), 426-441.

Meunier, M., Bachevalier, J., Mishkin, M., and Murray, E. A. (1993). Effects on visual recognition of combined and separate ablations of the entorhinal and perirhinal cortex in rhesus monkeys. *The Journal of Neuroscience, 13*(12), 5418-5432.

Milner, A. D., and Goodale, M. A. (2008). Two visual systems re-viewed. *Neuropsychologia, 46*(3), 774-785.

Mishkin, M., Suzuki, W. A., Gadian, D. G., and Vargha–Khadem, F. (1997). Hierarchical organization of cognitive memory. Philosophical Transactions of the Royal Society of London. *Series B: Biological Sciences, 352*(1360), 1461-1467.

Mitchell, K. J., and Johnson, M. K. (2009). Source Monitoring 15 Years Later: What Have We Learned From fMRI About the Neural Mechanisms of Source Memory? *Psychological Bulletin*, *135*(4), 638-677.

Montaldi, D., and Mayes, A. R. (2011). Familiarity, recollection and medial temporal lobe function: an unresolved issue. *Trends in Cognitive Sciences*, *15*(8), 339-340.

Montaldi, D., Spencer, T. J., Roberts, N., and Mayes, A. R. (2006). The neural system that mediates familiarity memory. *Hippocampus, 16*(5), 504-520.

Moscovitch, M. (2008). The Hippocampus As a "Stupid," Domain-Specific Module: Implications for Theories of Recent and Remote Memory, and of Imagination. *Canadian Journal of Experimental Psychology*, *62*(1), 62-79.

Moscovitch, M., Rosenbaum, R. S., Gilboa, A., Addis, D. R., Westmacott, R., Grady, C., et al. (2005). Functional neuroanatomy of remote episodic, semantic and spatial memory: a unified account based on multiple trace theory. *Journal of Anatomy*, *207*(1), 35-66.

Mumby, D. G. (2001). Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. *Behavioural Brain Research*, *127*(1-2), 159-181.

Mumby, D. G., and Pinel, J. P. J. (1994). Rhinal cortex lesions and object recognition in rats. *Behavioral Neuroscience*, 108(1), 11-18.

Nadel, L., and Hardt, O. (2011). Update on Memory Systems and Processes. *Neuropsychopharmacology, 36*, 251-273.

Nadel, L., and Moscovitch, M. (1997). Memory consolidation, retrograde amnesia and the hippocampal complex. *Current Opinion in Neurobiology*, *7*(2), 217-227.

Naya, Y., and Suzuki, W. A. (2011). Integrating What and When Across the Primate Medial Temporal Lobe. *Science, 333*(6043), 773-776.

Norman, G., and Eacott, M. J. (2005). Dissociable effects of lesions to the perirhinal cortex and the postrhinal cortex on memory for context and objects in rats. *Behavioral Neuroscience*, *119*(2), 557-566.

Norman, K. A., Polyn, S. M., Detre, G. J., and Haxby, J. V. (2006). Beyond mind-reading: multi-voxel pattern analysis of fMRI data. *Trends in Cognitive Sciences, 10*(9), 424-430.

Nyberg, L. (2005). Any novelty in hippocampal formation and memory? *Current Opinion in Neurology*, *18*(4), 424-428.

O'Keefe, J., and Nadel, L. (1978). The Hippocampus as a Cognitive Map. Oxford: Oxford University Press.

Otto, T., and Eichenbaum, H. (1992). Neuronal activity in the hippocampus during delayed non-match to sample performance in rats: Evidence for hippocampal processing in recognition memory. *Hippocampus*, *2*(3), 323-334.

Patterson, K., Nestor, P. J., and Rogers, T. T. (2007). Where do you know what you know? The representation of semantic knowledge in the human brain. *Nature Reviews Neuroscience*, *8*(12), 976-987.

Ploner, C. J., Gaymard, B. M., Rivaud-Péchoux, S., Baulac, M., Clémenceau, S., Samson, S., et al. (2000). Lesions affecting the parahippocampal cortex yield spatial memory deficits in humans. *Cerebral Cortex, 10*(12), 1211-1216.

Poppenk, J., McIntosh, A. R., Craik, F. I. M., and Moscovitch, M. (2010). Past Experience Modulates the Neural Mechanisms of Episodic Memory Formation. *The Journal of Neuroscience*, *30*(13), 4707-4716.

Quamme, J. R., Yonelinas, A. P., and Norman, K. A. (2007). Effect of unitization on associative recognition in amnesia. *Hippocampus, 17*(3), 192-200.

Rekkas, P. V., and Constable, R. T. (2005). Evidence that autobiographic memory retrieval does not become independent of the hippocampus: an fMRI study contrasting very recent with remote events. *Journal of Cognitive Neuroscience, 17*(12), 1950-1961.

Rempel-Clower, N. L., and Barbas, H. (2000). The Laminar Pattern of Connections between Prefrontal and Anterior Temporal Cortices in the Rhesus Monkey is Related to Cortical Structure and Function. *Cerebral Cortex*, *10*(9), 851-865.

Roediger, H. L. I. (1990). Implicit Memory: Retention Without Remembering. *American Psychologist*, *45*(9), 1043-1056.

Rosenbaum, R., Priselac, S., Kohler, S., Black, S., Gao, F., Nadel, L., et al. (2000). Remote spatial memory in an amnesic person with extensive bilateral hippocampal lesions. *Nature Neuroscience*, *3*(10), 1044-1048.

Rosenbaum, R. S., Ziegler, M., Winocur, G., Grady, C. L., and Moscovitch, M. (2004). "I have often walked down this street before": fMRI Studies on the hippocampus and other structures during mental navigation of an old environment. *Hippocampus, 14*(7), 826-835.

Ryan, L., Lin, C.-Y., Ketcham, K., and Nadel, L. (2010). The role of medial temporal lobe in retrieving spatial and nonspatial relations from episodic and semantic memory. *Hippocampus*, *20*(1), 11-18.

Ryan, L., Nadel, L., Keil, K., Putnam, K., Schnyer, D., Trouard, T., et al. (2001). Hippocampal complex and retrieval of recent and very remote autobiographical memories: Evidence from functional magnetic resonance imaging in neurologically intact people. *Hippocampus, 11*(6), 707-714.

Sagar, S. M., Sharp, F. R., and Curran, T. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science, 240*(4857), 1328-1331.

Saksida, L. M., Bussey, T. J., Buckmaster, C. A., and Murray, E. A. (2006). No effect of hippocampal lesions on perirhinal cortex dependent feature ambiguous visual discriminations. *Hippocampus, 16*(4), 421-430.

Sauvage, M. M., Fortin, N. J., Owens, C. B., Yonelinas, A. P., and Eichenbaum, H. (2008). Recognition memory: Opposite effects of hippocampal damage on recollection and familiarity. *Nature Neuroscience, 11*(1), 16-18.

Schott, B. H., Wüstenberg, T., Wimber, M., Fenker, D. B., Zierhut, K. C., Seidenbecher, C. I., et al. (2013). The relationship between level of processing and hippocampal–cortical functional connectivity during episodic memory formation in humans. *Human Brain Mapping*, *34*(2), 407-424.

Scoville, W. B., and Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of Neurology, Neurosurgery and Psychiatry*, *20*(1), 11-21.

Shing, Y. L., Werkle-Bergner, M., Li, S. C., and Lindenberger, U. (2008). Associative and strategic components of episodic memory: A life-span dissociation. *Journal of Experimental Psychology: General*, *137*(3), 495-513.

Shrager, Y., Kirwan, C. B., and Squire, L. R. (2008). Activity in both hippocampus and perirhinal cortex predicts the memory strength of subsequently remembered information. *Neuron, 59*(4), 547-553.

Simons, J. S., and Spiers, H. J. (2003). Prefrontal and medial temporal lobe interactions in long-term memory. *Nature Reviews Neuroscience*, *4*(8), 637-648.

Söderlund, H., Moscovitch, M., Kumar, N., Mandic, M., and Levine, B. (2012). As time goes by: Hippocampal connectivity changes with remoteness of autobiographical memory retrieval. *Hippocampus*, *22*(4), 670-679.

Squire L. R., Cohen N. J., Nadel L. (1984) The medial temporal region and memory consolidation: A new hypothesis. *Memory consolidation*, 185-210.

Squire, L. R., and Alvarez, P. (1995). Retrograde amnesia and memory consolidation: a neurobiological perspective. *Current Opinion in Neurobiology*, *5*(2), 169-177.

Squire, L. R., Stark, C. E. L., and Clark, R. E. (2004). The medial temporal lobe. *Annual Reviews Neuroscience*, *27*, 279-306.

Squire, L. R., Wixted, J. T., and Clark, R. E. (2007). Recognition memory and the medial temporal lobe: A new perspective. Nature Reviews. *Neuroscience*, *8*(11), 872-883.

Squire, L. R., and Zola, S. M. (1996). Structure and function of declarative and nondeclarative memory systems. *Proceedings of the National Academy of Sciences, 93*(24), 13515-13522.

Staresina, B. P., and Davachi, L. (2006). Differential Encoding Mechanisms for Subsequent Associative Recognition and Free Recall. *The Journal of Neuroscience, 26*(36), 9162-9172.

Staresina, B. P., and Davachi, L. (2009). Mind the Gap: Binding Experiences across Space and Time in the Human Hippocampus. *Neuron, 63*(2), 267-276.

Suddendorf, T., and Corballis, M. C. (1997). Mental time travel and the evolution of the human mind. *Genetic, Social, and General Psychology Monographs, 123*, 133-167.

Suzuki, W. L., and Amaral, D. G. (1994). Perirhinal and parahippocampal cortices of the macaque monkey: Cortical afferents. *The Journal of Comparative Neurology, 350*(4), 497-533.

Teng, E., and Squire, L. R. (1999). Memory for places learned long ago is intact after hippocampal damage. *Nature, 400*, 675-677.

Teyler, T. J., and DiScenna, P. (1986). The Hippocampal Memory Indexing Theory. *Behavioral Neuroscience*, *100*(2), 147-154.

Thomas, B. P., Welch, E. B., Niederhauser, B. D., Whetsell, W. O., Anderson, A. W., Gore, J. C., et al. (2008). High-resolution 7T MRI of the human hippocampus in vivo. *Journal of Magnetic Resonance Imaging*, *28*(5), 1266-1272.

Thompson, R. F., and Krupa, D. J. (1994). Organization of memory traces in the mammalian brain. *Annual Review of Neuroscience*, *17*(1), 519-549.

Tsao, D. Y., Cadieu, C. F., and Livingstone, M. S. (2010). Chapter 24. Object Recognition: Physiological and Computational Insights. *Primate Neuroethology, 1*(9), 471-500.

Tse, D., Langston, R. F., Kakeyama, M., Bethus, I., Spooner, P. A., Wood, E. R., et al. (2007). Schemas and Memory Consolidation. *Science, 316*(5821), 76-82.

Tulving, E. (1972). Episodic and Semantic Memory. New York: Academic Press.

Tulving, E. (1986). What Kind of a Hypothesis Is the Distinction Between Episodic and Semantic Memory? *Journal of Experimental Psychology: Learning, Memory, and Cognition, 12*(2), 307-311.

Ungerleider, L. G., and Haxby, J. V. (1994). 'What'and 'where' in the human brain. *Current Opinion in Neurobiology, 4*(2), 157-165.

Uylings, H., Groenewegen, H. J., and Kolb, B. (2003). Do rats have a prefrontal cortex? *Behavioural Brain Research*, *146*(1-2), 3-17.

van Goethem, N. P., Rutten, K., van der Staay, F. J., Jans, L. A. W., Akkerman, S., Steinbusch, H. W. M., Blokland, A., van 't Klooster, J., and Prickaerts, J. (2012). Object recognition testing: Rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research*, *232*(2), 323-334.

van Kesteren, M. T. R., Fernández, G., Norris, D. G., and Hermans, E. J. (2010). Persistent schema-dependent hippocampal-neocortical connectivity during memory encoding and postencoding rest in humans. *Proceedings of the National Academy of Sciences*, *107*(16), 7550-7555.

van Strien, N. M., Cappaert, N. L. M., and Witter, M. P. (2009). The anatomy of memory: an interactive overview of the parahippocampal–hippocampal network. *Nature Reviews Neuroscience*, *10*(4), 272-282.

Wais, P. E., Wixted, J. T., Hopkins, R. O., and Squire, L. R. (2006). The hippocampus supports both the recollection and the familiarity components of recognition memory. *Neuron*, *49*(3), 459-466.

Wan, H., Aggleton, J. P., and Brown, M. W. (1999). Different Contributions of the Hippocampus and Perirhinal Cortex to Recognition Memory. *The Journal of Neuroscience, 19*(3), 1142-1148.

Wang, S. H., and Morris, R. G. M. (2010). Hippocampal-neocortical interactions in memory formation, consolidation, and reconsolidation. *Annual Review of Psychology*, *61*, 49-79.

Warburton, E., and Brown, M. W. (2010). Findings from animals concerning when interactions between perirhinal cortex, hippocampus and medial prefrontal cortex are necessary for recognition memory. *Neuropsychologia, 48*(8), 2262-2272.

Wilson, C. R. E., Baxter, M. G., Easton, A., and Gaffan, D. (2008). Addition of fornix transection to frontal temporal disconnection increases the impairment in object in place memory in macaque monkeys. *European Journal of Neuroscience, 27*(7), 1814-1822.

Wilson, C. R. E., Gaffan, D., Browning, P. G. F., and Baxter, M. G. (2010). Functional localization within the prefrontal cortex: missing the forest for the trees? *Trends in Neurosciences*, *33*(12), 533-540.

Wiltgen, B. J., and Tanaka, K. Z. (2013). Systems consolidation and the content of memory. *Neurobiology of Learning and Memory*, *106*, 365-371.

Winocur, G., and Moscovitch, M. (2011). Memory Transformation and Systems Consolidation. *Journal of the International Neuropsychological Society, 17*(05), 766-780.

Winocur, G., Moscovitch, M., and Sekeres, M. (2007). Memory consolidation or transformation: context manipulation and hippocampal representations of memory. *Nature Neuroscience*, *10*(5), 555-557.

Winters, B. D., and Bussey, T. J. (2005). Transient inactivation of perirhinal cortex disrupts encoding, retrieval, and consolidation of object recognition memory. *The Journal of Neuroscience*, *25*(1), 52-61.

Winters, B. D., Saksida, L. M., and Bussey, T. J. (2008). Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neuroscience and Biobehavioral Reviews*, *32*(5), 1055-1070.

Wixted, J. T., and Squire, L. R. (2011a). The familiarity/recollection distinction does not illuminate medial temporal lobe function: response to Montaldi and Mayes. *Trends in Cognitive Sciences*, *15*(8), 340-341.

Wixted, J. T., and Squire, L. R. (2011b). The medial temporal lobe and the attributes of memory. *Trends in Cognitive Sciences*, *15*(5), 200-217.

Xiang, J. Z., and Brown, M. W. (1998). Differential neuronal encoding of novelty, familiarity and recency in regions of the anterior temporal lobe. *Neuropharmacology, 37*(4-5), 657-676.

Yonelinas, A. P. (2001). Components of episodic memory: The contribution of recollection and familiarity. Philosophical Transactions of the Royal Society of London. *Series B: Biological Sciences*, *356*(1413), 1363-1374.

Yonelinas, A. P. (2002). The Nature of Recollection and Familiarity: A Review of 30 Years of Research. *Journal of Memory and Language, 46*(3), 441-517.

Yonelinas, A. P., Kroll, N. E. A., Quamme, J. R., Lazzara, M. M., Sauve, M., Widaman, K. F., et al. (2002). Effects of extensive temporal lobe damage or mild hypoxia on recollection and familiarity. *Nature Neuroscience*, *5*(11), 1236-1241.

Yonelinas, A. P., Otten, L. J., Shaw, K. N., and Rugg, M. D. (2005). Separating the brain regions involved in recollection and familiarity in recognition memory. *The Journal of Neuroscience*, *25*(11), 3002-3008.

Zola-Morgan, S., Squire, L., Amaral, D., and Suzuki, W. (1989). Lesions of perirhinal and parahippocampal cortex that spare the amygdala and hippocampal formation produce severe memory impairment. *The Journal of Neuroscience, 9*(12), 4355-4370.

Chapter 3

Object Recognition Testing: Rodent species, strains, housing conditions and estrous cycle

Nick P. van Goethem, Kris Rutten, Franz Josef van der Staay, Linda A.W. Jans, Sven Akkerman, Harry W.M. Steinbusch, Arjan Blokland, José van 't Klooster and Jos Prickaerts

Behavioural Brain Research (2012), 232(2), 323-334

Abstract

The object recognition task (ORT) allows assessing learning and memory processes in rodents. In this study, two areas in which knowledge about the ORT could be extended were addressed; i.e. generality to species and strains, and intervening variables including housing and estrous cycle. Regarding generality to species and strains, the ORT performance of golden hamsters was assessed. The hamsters showed sufficient exploration times, object recognition performance, and a retention-interval dependent decline similar to rats and mice. Subsequently, we tested three mouse strains which have not been described before in the ORT; i.e. OF1, NMRI, and SJL mice. OF1 and NMRI strains performed equally well, whereas the SJL strain showed low exploration times and no memory retention. Therefore, the SJL strain is unsuited for ORT experiments using a 1 h retention interval and a fixed (3 min) trial duration. Furthermore, the sensitivity to a pharmacological memory deficit model (scopolamine) was tested in three rat strains. Each strain showed a dose dependent relationship, but the least effective dose of scopolamine differed among the three strains, the effect being greater in the order of Wistar, Long-Evans, Hooded Lister rats. Finally, to investigate potential intervening variables in the ORT, the effects of housing conditions and estrous cycle were investigated with rats. Single housing resulted in absolute higher performance than social housing. Furthermore, females in pro-estrus/estrus showed better performance

compared to females in met-estrus/di-estrus. Taken together, object recognition appears to be a common ability of rodent species, but different strains have different memory capacities and sensitivities to scopolamine, individual housing leads to higher performance and performance of females is dependent on the estrous cycle phase. Thus, rodent species, strain, housing, and estrous cycle should be taken into consideration in ORT studies.

Introduction

The object recognition task (ORT) is based on 'spontaneous novelty preference', i.e. the natural predisposition of rodents to explore novel objects (Mumby et al., 2002). This task consists of two trials, one acquisition trial, followed by one retention trial after an inter-trial interval ranging from minutes to hours. Because one acquisition trial suffices, the ORT is characterised as a one-trial learning paradigm. Different rodent species appear to perform at a similar level in this task (e.g. rattus norvegicus: (Ennaceur and Delacour, 1988; Prickaerts et al., 2002b); mus musculus: (Dodart et al., 1997; Messier, 1997); peromyscus californicus: (Bredy et al., 2004); phodopus sungorus: (Palchykova et al., 2006)) and therefore, the ability to discriminate between familiar and novel objects may be a common ability among rodent species. However, in order to obtain reliable estimates of ORT performance, the animals tested should show a sufficiently high level of spontaneous object exploration (Şik et al., 2003), and show good retention performance after a relatively short retention interval, e.g. 1 h (Dodart et al., 1997; Şik et al., 2003). The ORT allows assessing the effects of experimental interventions, such as lesioning of selected brain areas (Mumby et al., 2002), genetic manipulations (Vaucher et al., 2002), administration of compounds that impair recognition performance (Dodart et al., 1997; Norman et al., 2002), or of putative cognition enhancers (Ennaceur et al., 1989; Norman et al., 2002; Prickaerts et al., 2002a, 2002b) on learning and memory processes in rodents.

Although the ORT seems a simple and straightforward paradigm, many researchers have communicated difficulties in establishing this task in their laboratories (personal communications). The present studies aimed to further extend on several features and aspects of the ORT that should be taken into account and which may affect or even confound the outcome measures of this task. We addressed two areas in which knowledge about the ORT could be extended. These areas included; generality to species and strains, and intervening variables including housing and estrous cycle in the ORT.

With respect to generality, the knowledge of whether object recognition is a common ability among rodent species (and strains) was extended by means of testing golden (Syrian) hamsters (Mesocricetus auratus) for this feature. In relation to memory, golden hamsters are a species which have been used for research in the area of circadian rhythms (Cain and Ralph, 2009), but also for investigating the neural regulation of social recognition due to their high reliance on odor cues (Petrulis, 2009). To our knowledge this species has never been described for object recognition performance. In the present study, special

attention was paid to the question whether these animals showed a sufficient level of spontaneous object exploration, and whether they showed above-chance object recognition, at least after a short retention interval. Both are prerequisites to use either drug-induced (e.g. Dodart et al., 1997; Ennaceur and Meliani, 1992b; Lieben et al., 2005) or delay-induced poor discrimination performance for testing the efficacy of putative cognition enhancers (e.g. Norman et al., 2002; Prickaerts et al., 2002a, 2002b) in the ORT. To further extend basic knowledge of generality in the ORT, the usefulness of three different mouse strains, which have not been described previously in the ORT, were assessed. Earlier research indicates that different mouse strains show dissimilar ORT performance (e.g. Şik et al., 2003). We tested the (baseline) ORT performance of the outbred OF1 and NMRI, and the inbred SJL mouse strains, which are used routinely in drug finding studies (e.g. for OF1 mice (Bour et al., 2004); for NMRI mice (Kathmann et al., 2001); and for SJL mice (Griebel et al., 2000)).

In conjunction with the first area we addressed, a second aim concerned strain sensitivity to a commonly used pharmacological memory deficit model in the ORT, i.e. administration of the anticholinergic drug scopolamine. The muscarinic antagonist scopolamine is widely used as a memory-deficit-inducing compound in behavioral and pharmacological studies (e.g. Ennaceur and Meliani, 1992b; Norman et al., 2002). A variety of studies investigated the effects of cholinergic depletion on cognition in different rat strains. These studies led to varying results, with inconsistent conclusions regarding the detrimental effects of cholinergic depletion on the cognitive performance of different rat strains (e.g. Higashida and Ogawa, 1987; Andrews et al., 1995; Bushnell et al., 1995; Gleason et al., 1999). Therefore, the effects of cholinergic blockade in different rat strains were investigated by means of testing three male outbred rat strains, which have been used the ORT before (Long-Evans, Hooded Lister, and Wistar rats), with regard to the impact of different doses of scopolamine on ORT performance. The aim of this experiment was to investigate, per rat strain separately, whether there were differences in the lowest dose of scopolamine needed to induce memory-deficits in the ORT.

With regard to the second area of intervening variables in the ORT, two experiments were conducted in which the effects of housing conditions and the effects of sex and estrous cycle phases in Wistar rats were investigated. In the first experiment, the differences in ORT performance between individually and socially housed male Wistar rats were assessed. Since rats are social animals (Meaney and Stewart, 1981), the standard housing conditions for laboratory rats

is social housing, i.e. the rats are housed in groups which vary in size. It was shown that housing conditions have the potential to influence the ORT performance of male Sprague-Dawley rats (Beck and Luine, 2002). In order to investigate the effects of single and social housing conditions on the ORT performance of male Wistar rats, animals were either housed socially in groups of three, or individually. Subsequently, the ORT performance of these two groups was assessed and compared. Wistar rats were chosen since this strain is commonly used in behavioral and pharmacological studies (e.g. Andrews et al., 1995; Prickaerts et al., 2002a, 2002b; Lieben et al., 2005); hence it is important to be aware of factors that could influence their ORT results. In the second experiment the effects of sex and estrous cycle phase on object recognition memory in Wistar rats was investigated. In agreement with human data (Delgado and Prieto, 1996; Collins and Kimura, 1997), rodents also show sex differences: males tend to outperform females in spatial memory tasks (Jonasson, 2005; Blokland et al., 2006), while females show a better performance in tasks assessing object recognition (Ghi et al., 1999). Because of the fluctuations of female hormone levels observed during the estrous cycle, estrous cycle phase may be an important factor when working with female animals (Ennaceur et al., 2005; Singletary et al., 2005). Obviously, ovarian steroid action in the brain is not limited to reproductive effects and behaviors. Research performed on humans and rodents during the past decade, confirms that gonadal steroids (e.g. oestradiol) affect structural properties of brain regions that subserve learning and memory (e.g. the cerebral cortex and hippocampus) (Sutcliffe et al., 2007). If females differ in memory performance from males on some (but not all) days of the estrous cycle, and if experimental groups are created at random, females may not be randomly distributed across the estrous cycle, which could thus confound the outcome of the experiment**.** The ORT performance of two groups of female rats (female rats in pro-estrus/estrus and female rats in met-estrus/di-estrus) and a group of male rats was compared in order to assess the effect of sex and estrous cycle phase.

In summary, two areas in which knowledge about the ORT could be extended were addressed in this study. These areas included generality to species and strains (including rat strain sensitivity to a pharmacological memory deficit model), and intervening variables including housing and estrous cycle in the ORT.

Material and methods

All procedures were designed to minimize the potential discomfort of the animals during the behavioral experiments. All experimental procedures were

approved by the local ethical committee for animal experiments according to governmental guidelines.

Species and strain generality in object recognition testing

Object recognition in golden hamsters (Mesocricetus auratus)

Animals

A total of five 2-3-months-old female golden (Syrian) hamsters (Harlan, Horst, The Netherlands) were used for this experiment. The animals were housed individually in standard Makrolon™ type IV cages on sawdust bedding. Nestlets™ were provided as nesting material. The room temperature was about 20°C (and 60 ± 10% relative humidity). The animals were kept under a reversed 10/14-h light/dark cycle (lights on from 21:00 to 7:00h) and food and water was available ad libitum. During the dark period, when testing took place, fluorescent red tubes provided a constant illumination (2 lux). The hamsters were housed in the same room where the testing took place. The testing apparatus was always at least at two meters distant from the home cages, which were placed behind the apparatus. During testing the test room was dimly lit by a small lamp (25W), located in the corner of the room.

Object recognition memory

The ORT for hamsters was performed in the same way as earlier described for mice (Dodart et al., 1997; Messier, 1997). The apparatus consisted out of a circular arena which was 48 cm in diameter, with a 40 cm high wall made of transparent polyvinyl chloride. During testing, the arena was dimly illuminated by a small lamp (25W), located in the corner of the room dimmed with a white paper filter. This resulted in a light intensity of approximately 8 lux which was equal in the different parts of the arena. In the ORT trials, two objects were placed in the arena in a symmetrical position about 5 cm away from the wall.

In each trial the objects were placed on the exact same location. Four different sets of objects were used. Each object was available in triplicate so that a hamster did not get to explore an object it already explored in the first (sample) trial (T1). This means that in the second (test) trial (T2) an exact replica of an earlier explored object was presented to the hamster. This way, potential traces that could attract a hamster towards the familiar object were excluded. Hence, the observed exploratory behavior was solely based on familiarity instead of other factors. The different sets of objects used were: (1) a little glass bottle (diameter 8.0 cm, height 9.6 cm), (2) a plastic cup (diameter 8.0 cm, height 9.6

cm), (3) a block made of Duplo™ (8.0 cm x 6.3 cm x 6.3 cm), (4) a pyramid made of Duplo™ (height 8.0 cm, base 6.3 cm x 6.3 cm). The objects could not be displaced by a hamster. Furthermore, the objects had no natural significance for hamsters nor had they ever been associated with reinforcement.

In the weeks preceding the ORT, the hamsters were extensively handled. They were used to evaluate the applicability of a series of sensorimotor tests that are routinely being used in mice and rats. In the period preceding the ORT experiment, the hamsters were handled daily and were adapted to the arena (without any objects) twice for 5 min each day. ORT objects familiarization sessions were not performed, i.e. the animals were not familiarized to the ORT objects before the actual testing started. Because of this the objects were entirely new when the hamsters first encountered them. Normally a slightly better performance is seen when animals encounter objects for the first time than when they are familiarized with the objects (Akkerman et al., 2012). The purpose of the study described here was solely to investigate whether golden hamsters were able to recognize objects and hence show sufficient object recognition. After habituation to the testing arena during two sessions, ORT performance of the hamsters was tested after retention intervals of 10 min, 60 min, 4 h, and 24 h (this was also the order of testing). This order of testing was repeated once, so the animals were tested twice at each of the four retention intervals, after which the data was pooled in order to increase power. This was necessary because of the small sample size (n = 5). The testing was done with an inter-test interval of 24 h between T1 and T2 pairs at the two short retention intervals (10 and 60 min), and with an inter-test interval of 48 h between T1 and T2 pairs at the two longer retention intervals (4 and 24 h). After all four intervals were tested for the first time, 48 h was interposed before all four intervals were tested for the second time.

An ORT testing session comprised two trials (T1 and T2) and the duration of each trial was 3 min. During T1 the arena contained two identical sample objects. A hamster was always placed in the arena facing the wall at the middle of the front segment. After the first exploration period the hamster was put back in its home cage, which was located behind the back segment of the apparatus at least at two meters distant. Subsequently, after the retention interval, the hamster was put back into the arena for T2, but now with two dissimilar objects, a familiar one (a replica of the sample) and a new one. A schematic representation of this procedure is found in Figure 1. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer.

Exploration of an object was defined as follows: directing the nose to an object at a distance of no more than 2 cm and/or touching an object with the nose. Sitting on, or leaning to, an object were not considered to be exploratory behavior (Ennaceur and Delacour, 1988; Prickaerts et al., 1997).

Since rodents can discriminate between objects based on olfactory cues (Astur et al., 2002), the objects were thoroughly cleaned after each trial with a 70% ethanol solution. All combinations and locations (left and right) of the objects were used in a balanced manner in order to reduce potential biases due to preferences for particular locations or objects.

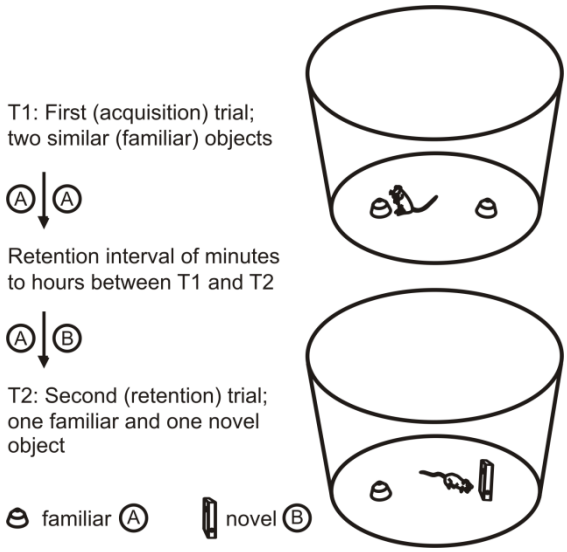


Figure 1. The object recognition task. Schematic presentation of the test procedure of the ORT.

Object recognition in different strains of mice

Animals

A total of ten 2-months-old male outbred OF1 mice (Crl:OF1), ten 2-months-old male outbred NMRI mice (Crl:NMRI(Han)), and ten 2-months-old male inbred SJL mice (SJL/JOrlCrl) (Iffa Credo; now Charles River, Sulzfeld, Germany) were used for this experiment. The OF1 and the NMRI mice were housed in groups of 10 in Makrolon™ type III cages on sawdust bedding. In the same room, the SJL mice were housed individually in Makrolon™ type II cages, because of aggressive behavior. The room temperature was about 20°C (and 60 ± 10% relative humidity). The animals were kept under a normal 12/12-h light/dark cycle (lights on from 6:00 to 18:00h) and food and water was available ad libitum. The mice were housed in the same room where the testing took place. The testing apparatus was always at least at two meters distant from the home cages, which were placed behind the apparatus. All testing was done between 7:00 and 12:00.

Object recognition memory

The same apparatus and procedures as in the hamster experiment were used (see *paragraph 2.1.1.2.*). Because of testing during the light phase, the light intensity was approximately 100 lux and was equal in the different parts of the arena. The objects used for the mice differed from the objects used in the hamster study. For the mice the following objects were used in the ORT: (1) a brass rod with a tapering top (diameter 3.0 cm, height 11.5 cm), (2) a massive metal cube (7.5 cm x 5.0 cm x 2.5 cm) with two holes (diameter 1.5 cm) in it, (3) a white plastic cube (7.0 cm x 5.0 cm x 5.0 cm) with rounded edges and a circular foot (diameter 4.5 cm), (4) a massive aluminium cube with a tapering top (9.0 cm x 4.5 cm x 4.5 cm). Furthermore, the mice were only tested in one retention interval, i.e. 1 h, to assess their baseline performance. In order to increase exploratory activity in the ORT, a mouse was put into an empty cage for 3 min immediately preceding each of the trials. The ORT was repeated once, with a 48 h interval between pairs of trials. This was done in order to have more observations since there was a relative small sample size (n = 10 per strain). Subsequently, like in the hamster experiment, the data of both tests was pooled in order to increase power.

Memory impairing effects of scopolamine in different strains of rats

Animals

A total of twelve 3-months-old male Hooded Lister rats (Charles River, Margate, United Kingdom), twelve 3-months-old male Long-Evans rats (Charles River, Sulzfeld, Germany), and twelve 3-months-old male Wistar rats (Harlan, Horst, The Netherlands) were used for this experiment. One of the Hooded Lister rats

died during the early stages of the experiment due to reasons unknown. The animals were housed individually in standard Makrolon™ type III cages on sawdust bedding. The room temperature was about 20°C (and 60 ± 10% relative humidity). The animals were kept under a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00h) and food and water was available ad libitum. During the dark period, when testing took place, fluorescent red tubes provided a constant illumination (2 lux). The rats were housed in the same room where the testing took place. The testing apparatus was always at least at two meters distant from the home cages, which were placed behind the apparatus. During testing the test room was dimly lit by a small lamp (25W), located in the corner of the room.

Treatment

Scopolamine hydrobromide (Acros organics, Belgium) was prepared daily (dissolved in saline), and was always administered intraperitoneal (i.p.) 30 min before T1. The tested doses were; 0.03, 0.1, and 0.3 mg/kg scopolamine. In order to investigate whether the animals showed sufficient (baseline) object recognition, saline conditions were included for all three strains. After testing of the saline conditions, the experimental scopolamine conditions were tested. First, a dose-finding study in which all three scopolamine doses were tested was performed in Wistar rats. On each testing day, every scopolamine condition was tested (n = 4 per scopolamine condition per testing day), so the order of testing was counterbalanced. From these data the optimal dose was determined which was the starting-point for the two other strains. Both the Long-Evans and the Hooded Lister rats were treated with this optimal dose of scopolamine in a number of animals (n = 4), and from these outcomes it was decided whether to add a higher or a lower dose condition. Again, the order of scopolamine dosage administration was balanced in order to rule out any test order effects. Due to this design, not each of the three scopolamine doses had to be tested in both Long-Evans and Hooded Lister rats, and hence less i.p. injections had to be administrated to the rats. It was investigated at which dose object recognition (discrimination indices) ceased to be significantly different from zero (or chance level), and hence no recognition was present (Prickaerts et al., 1997). This was investigated per rat strain separately. Since not every strain received each drug condition, comparing doses effects between rat strains was not feasible. A between analyses comparison (one-way ANOVA) was performed only for the saline condition in order to rule out baseline strain differences in object recognition. In addition, every scopolamine condition was compared to the saline condition within each strain.

Object recognition memory

The ORT for rats was performed as described elsewhere (Ennaceur and Delacour, 1988; Prickaerts et al., 1997). The apparatus consisted out of a circular arena which was 83 cm in diameter. The floor of the arena and half of the 40 cm high wall was made of grey (RAL 7035) polyvinyl chloride, the other half of transparent polyvinyl chloride. During testing, the arena was dimly illuminated by a small lamp (25W), located in the corner of the room dimmed with a white paper filter. This resulted in a light intensity of approximately 8 lux which was equal in the different parts of the arena.

Two objects were placed in a symmetrical position about 10 cm away from the wall. In each trial the objects were placed on the exact same location. Four different sets of objects were used. Each object was available in triplicate so that a rat did not get to explore an object it already explored in T1. This means that in T2 an exact replica of an earlier explored object was presented to the rat. This way, potential traces that could attract the rat towards the familiar object were excluded, hence the observed exploratory behavior was solely based on familiarity instead of other factors. The different sets of objects used were: (1) a standard 1 litre brown glass bottle (diameter 10.0 cm, height 22.0 cm) filled with water and closed with a black cap, (2) a massive metal cube (10.0 cm x 5.0 cm x 7.5 cm) with two holes (diameter 1.9 cm) in it, (3) a cone consisting of a grey polyvinyl chloride base (maximal diameter 18.0 cm) with a collar on top made of aluminium (total height 16.0 cm), (4) a massive aluminium cube with a tapering top (13.0 cm x 8.0 cm x 8.0 cm).

In the period preceding the ORT testing, rats were handled daily and were adapted to the arena (without any objects) twice for 5 min per day. After this habituation, ORT objects familiarization sessions were performed, i.e. the animals were familiarized to all the ORT objects before the actual testing started. Because of this the objects were no longer entirely new when the rats first encountered them. Normally a slightly better performance is seen when animals encounter objects for the first time than when they are familiarized with the objects (Akkerman et al., 2012). After this familiarization, the ORT performance of the rats stabilizes. In addition, the rats had to get habituated to the i.p. injections. Thus, after handling and habituation to the arena, ORT objects familiarization sessions were performed in combination with i.p. (saline 1.0 ml/kg) injections, to habituate to this procedure.

An ORT testing session comprised two trails (T1 and T2) and the duration of each trial was 3 min. During T1 the arena contained two identical objects (samples). A rat was always placed in the arena facing the wall at the middle of the front

(transparent) segment. After the first exploration period (T1) the rat was put back into its home cage which was located behind the back segment of the apparatus at least at two meters distant. Subsequently, after a retention interval of 1 h, the rat was put back into the arena for T2, but now with two dissimilar objects, a familiar one (a replica of the sample) and a novel one. A schematic representation of this procedure is found in Figure 1. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer. This test was repeated once, with at least a 48 h interval between two ORT testing sessions. This was done in order to have more observations since there was a relative small sample size (n = 12 per strain). Subsequently, the data of both tests was pooled in order to increase power.

Exploration of an object was defined as follows: directing the nose to an object at a distance of no more than 2 cm and/or touching an object with the nose. Sitting on, or leaning to, an object were not considered to be exploratory behavior (Ennaceur and Delacour, 1988; Prickaerts et al., 1997).

Since rodents can discriminate between objects based on olfactory cues (Astur et al., 2002), the objects were thoroughly cleaned after each trial with a 70% ethanol solution. All combinations and locations (left and right) of the objects were used in a balanced manner in order to prevent potential bias due to preferences for particular locations or objects. The order of testing was randomly determined. The experimenter was always blind to the conditions tested.

Intervening variables in object recognition testing

Object recognition in social and single housed rats

Animals

A total of twenty-four 4-months-old male Wistar rats (Charles River, Sulzfeld, Germany) were used for this experiment. Twelve of these animals were housed individually, and twelve were housed socially in groups of three, in standard Makrolon™ type III cages on sawdust bedding. The room temperature was about 20°C (and 60 ± 10% relative humidity). The animals were kept under a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00h) and food and water was available ad libitum. During the dark period, when testing took place, fluorescent red tubes provided a constant illumination (2 lux). The rats were housed in the same room where the testing took place. The testing apparatus was always at least at two meters distant from the home cages, which were placed behind the

apparatus. During testing the test room was dimly lit by a small lamp (25W), located in the corner of the room.

Object recognition memory
The ORT was performed as in the above described rat study (see *paragraph 2.1.3.3.*), with the only exception that these rats did not receive any injections and hence, were not adapted to these injection procedures. The order of testing was randomly determined.

The effects of sex and estrous cycle on object recognition in rats
Animals
A total of twelve 4-months-old male Wistar rats and twenty-four 4-months-old female Wistar rats (Charles River, Sulzfeld, Germany) were used for this experiment. The animals were housed individually in standard Makrolon™ type III cages on sawdust bedding. The room temperature was about 20°C (and 60 ± 10% relative humidity). The animals were kept under a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00h) and food and water was available ad libitum. During the dark period, when testing took place, fluorescent red tubes provided a constant illumination (2 lux). The rats were housed in the same room where the testing took place. The testing apparatus was always at least at two meters distant from the home cages, which were placed behind the apparatus. During testing the test room was dimly lit by a small lamp (25W), located in the corner of the room.

Estrous cycle phase
At two months of age, young female rats exhibit estrous cycles and ovulation every 4 to 5 days. The rat estrous cycle can be divided into four phases: pro-estrus (lasting approximately 12 h), estrus (9-15 h), met-estrus (14-18 h), and di-estrus (60-70 h) (Singletary et al., 2005). The ovarian cycle begins with the development of follicles in the ovary, this is the met-estrus phase. Oestradiol secretion from the ovaries increases gradually during this phase. Next is di-estrus or the follicular phase, which is followed by the preovulatory period. The preovulatory period is also known as pro-estrus in rats. Estrus is the phase of sexual receptivity and the actual period of ovulation, which occurs 10-12 h after pro-estrus as the result of rising oestradiol levels (Becker et al., 2005; Singletary et al., 2005).

To determine the estrous cycle phase of each female rat, vaginal smears were taken between 7:00 and 10:00 AM on all testing days. A plastic smear loop was inserted in the vaginal opening, gently rotated and withdrawn. The smear loop

was immediately rolled onto a glass slide and allowed to air dry. Slides were examined under a light microscope for the presence of nucleated epithelial cells, cornified epithelial cells, leukocytes, and mucus. Estrous cycle phase was determined using the following criteria; (1) pro-estrus: predominantly nucleated epithelial cells; (2) estrus: predominantly cornified epithelial cells; (3) met-estrus: cornified epithelial cells and leukocytes; (4) di-estrus: predominantly leukocytes, some nucleated epithelial cells and mucus (Singletary et al., 2005). The group of females was subdivided into two groups based on estrous cycle phase in order to investigate the effects of estrous cycle phase on ORT performance. Female rats in pro-estrus and estrus formed one group (pro/es; characterized by higher levels of oestradiol and progesterone), and the other group consisted of female rats in met-estrus and di-estrus (met/di; characterized by lower levels of oestradiol and progesterone). Similar subdivisions have been described in other studies investigating the role of estrous cycle in behavior (Contreras et al., 2000). In short, there were three experimental groups in this study: (1) males, (2) females in pro-estrus and estrus (pro/es), and (3) females in met-estrus and di-estrus (met/di).

Object recognition memory

The ORT was performed as in the above described rat study (see *paragraph 2.1.3.3.*). Exceptions to this description are that the rats were only tested once (with the exception of the ORT objects familiarization sessions) for the actual ORT experiment and no injections had to be given. The order of testing was randomly determined. The experimenter was always blind to the conditions tested.

Statistical analysis

The basic measures were the times spent by the animals exploring an object during T1 and T2. The times spent exploring the two identical objects in T1 will be represented by ‘a1’ and ‘a2’. The times spent in exploring the familiar and the novel object in T2 will be represented by ‘a3’ and ‘b’, respectively. From these exploration times the following variables could be calculated: e1=a1+a2, e2=a3+b, d1=b-a3, and d2=(b–a3)/e2 (see Table 1). e1 and e2 are measures of the total exploration time of both objects during T1 and T2, respectively. d1 and d2 are considered as index measures of discrimination between the familiar and the novel objects. Actually, d2 is a relative measure of discrimination corrected for exploratory activity (e2). Because of this correction the d2 index deserves our preference, since it makes this parameter clearer to compare between conditions (Akkerman et al., 2012). The d2 index can range from -1 to 1, with -1

indicating complete preference for the familiar object, 0 signifying no preference for either object, and 1 indicating complete preference for the novel object. Thus, there should be no differences in d2 indices between experiments with similar treatments at similar intervals.

In order to assess object recognition performance, one-sample *t*-statistics were performed. This way it could be assessed per treatment condition whether the d2 index differed significantly from zero (i.e. chance level). A significant positive difference from zero indicates recognition for the familiar object encountered during T2. Furthermore, the difference between exploration times of the familiar and novel object (within experimental conditions) during T2 was assessed with paired-samples *t*-statistics (data not shown, significance levels are given in the corresponding Figures). In addition, to further investigate the effects between experimental conditions (e.g. differences in: mouse strain, drug condition, housing condition and sex/estrous cycle phase on ORT performance) on the measures e1, e2 and d2, analyses of variance (one-way ANOVAs) were conducted. For the hamster study, the data were analysed using a repeated measures ANOVA with retention interval as within-subjects factor. In the study comparing memory impairing effects of scopolamine in different strains of rats, an additional one-way ANOVA was conducted between ORT performances (d2) in the saline/vehicle conditions of the three strains. This way it could be assessed whether the three rat strains differed with respect to their baseline ORT performance. The saline condition was used because this should reflect the 'natural' recognition of the rats (under the conditions of receiving an i.p. injection) without the influence of scopolamine on memory. Since not every rat strain received all scopolamine conditions, between-group comparisons could not adequately be performed. In case of a significant difference between conditions, post hoc analyses (Bonferroni *t*-tests) were performed ($\alpha = 0.05$).

Exploration	Discrimination
e1 = a1 + a2	d1= b – a3
e2 = a3 + b	d2= (b – a3) / e2

Table 1. Measures involved in the ORT. e1 is the measure of the time spent in exploring both identical objects (a1 and a2) during T1, and e2 is the measure of the time spent in exploring both the familiar (a3) and new object (b) in T2; d1 and d2 correspond to the ability to discriminate between the old and new object during the second trial. Of these two discrimination indices only d2 is corrected for exploration time during T2.

Results

Species and strain generality in object recognition testing

Object recognition in golden hamsters (Mesocricetus auratus)

One-way ANOVA revealed no differences between retention intervals on the level of exploration in T1 (e1, $F_{3,16}$ = 1.08; P = 0.388). During T2, the hamsters explored the objects less with increasing retention interval (e2, $F_{3,16}$ = 3.27; P = 0.049)**.** The times spent exploring both identical objects in T1 (e1) were (mean + SEM): 10 min, 33.4 sec (3.3); 60 min, 25.9 sec (3.8); 4 h, 26.9 sec (3.4); 24 h, 28.9 (2.4). The times spent exploring the familiar and novel objects in T2 are depicted in Figure 2A. In T2, the time spent exploring the familiar object was not affected by the duration of the retention interval ($F_{3,16}$ = 1.87; P = 0.189), whereas the time spent exploring the novel object decreased with increasing interval ($F_{3,16}$ = 3.92; P = 0.037).

The d2 indices for the different retention intervals are graphically presented in Figure 2B. There was a tendency for the d2 indices to decrease with increasing retention interval ($F_{3,16}$ = 3.11; P = 0.056). One-sample t-statistics confirmed that the discrimination performance was significantly different from zero at the retention intervals of: 10 min (t_4 = 5.39; P = 0.006), 60 min (t_4 = 5.45; P = 0.006), and 4 h (t_4 = 10.23; P = 0.001), but not at the 24 h interval (t_4 = 1.55; P = 0.196).

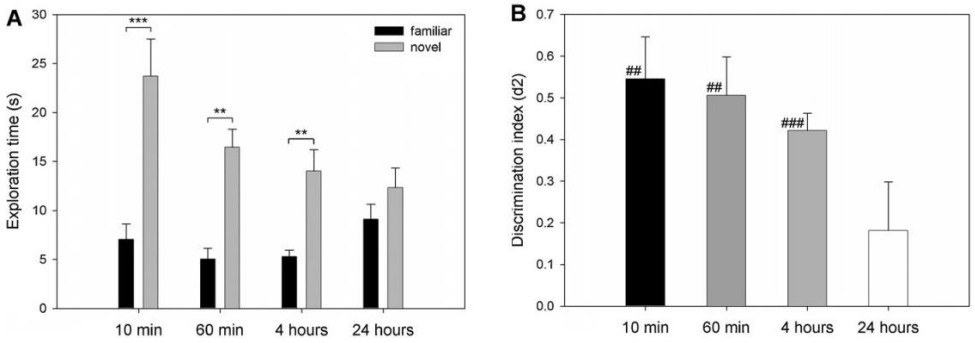


Figure 2. Object recognition in golden hamsters (Mesocricetus auratus). A) Exploration times of female golden (Syrian) hamsters for the familiar and novel object during T2 (mean + SEM) after different retention intervals. Significant differences in exploration times were found in all inter-trial retention intervals except in the 24 h retention interval, and are indicated with asterisks (Paired-samples t-tests, **: P < 0.01; ***: P < 0.001). **B)** Discrimination indices (d2) of the female golden (Syrian) hamsters (mean + SEM) after different retention intervals. There was a tendency for the d2 indices to decrease with increasing retention interval, although this decrease did not reach significance. A significant difference from zero (i.e. chance level) is indicated with hashes (One sample t-tests, ##: P < 0.01; ###: P < 0.001). n = 10 observations per experimental condition.

Object recognition in different strains of mice

An effect was found of experimental group on exploration times in T1 (e1, $F_{2,27}$ = 14.59; P = 0.000) and T2 (e2, $F_{2,27}$ = 17.80; P = 0.000). Post hoc comparisons confirmed that the SJL mice had less exploration time when compared to the OF1 (T1(e1): P = 0.001; T2(e2): P = 0.000) and NMRI (T1(e1): P = 0.000; T2(e2): P = 0.001) mice which did not differ from one another (T1(e1): P = 0.902; T2(e2): P = 0.458). The times spent exploring both identical objects in T1 (e1) were (mean + SEM): OF1 mice, 17.8 sec (2.5); NMRI mice, 20.6 sec (1.4); SJL mice, 6.9 (1.6). The exploration times per object in T2 are illustrated in Figure 3A.

The d2 index was affected by strain ($F_{2,27}$ = 5.46; P = 0.010). Post hoc analysis confirmed that the SJL mice had a lower d2 index than the OF1 (P = 0.035) and NMRI (P = 0.018) mice. One-sample t-statistics revealed that the d2 index of the OF1 (t_9 = 6.95; P = 0.000) and NMRI (t_9 = 11.87; P = 0.000) mice deviated from zero, whereas that of the SJL mice did not (t_9 = -0.23; P = 0.824) (see Figure 3B).

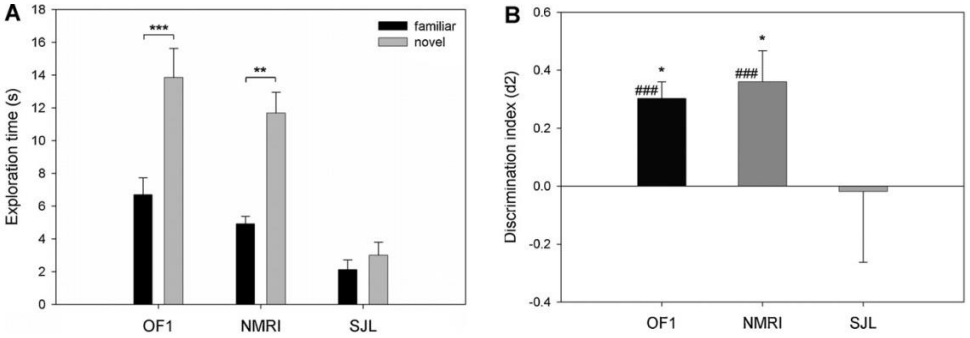


Figure 3. Object recognition in different strains of mice. A) Exploration times of the different mouse strains (OF1, NMRI and SJL mice) tested for the familiar and novel object during T2 (mean + SEM) after a 1 h retention interval. The exploration times of the familiar and novel objects only differed significantly in the OF1 and the NMRI strain. This is indicated with asterisks (Paired-samples t-tests, **: P < 0.01; ***: P < 0.001). **B)** Discrimination indices (d2) of the different mouse strains tested (mean + SEM). Significant differences between experimental groups were found. The NMRI and OF1 mice showed a higher discrimination index than the SJL mice. This is indicated with asterisks (Bonferroni t-tests, *: P < 0.05). A significant difference from zero (i.e. chance level) is indicated with hashes (One sample t-tests, ###: P < 0.001). n = 20 observations per experimental condition.

Memory impairing effects of scopolamine in different strains of rats

One-way ANOVA revealed no differences in the d2 indices between the three strains of rats in the saline conditions ($F_{2,64}$ = 0.99; P = 0.376). The times spent

exploring both identical objects in T1 (e1) for all three rat strains were (mean + SEM): Wistar rats: saline, 27.5 sec (2.3); 0.03 mg/kg scopolamine, 21.1 (1.3); 0.1 mg/kg scopolamine, 20.8 (1.6); 0.3 mg/kg scopolamine, 28.7 (2.1). Long-Evans rats: saline, 22.2 sec (1.4); 0.03 mg/kg scopolamine, 29.5 (2.1); 0.1 mg/kg scopolamine, 28.6 (2.1). Hooded Lister rats: saline, 22.3 sec (3.1); 0.1 mg/kg scopolamine, 21.6 (2.2); 0.3 mg/kg scopolamine, 26.1 (3.3). For all three rat strains, the times exploring the familiar and novel objects during T2 are graphically presented in Figure 4.

For the Wistar rats, an effect of drug condition was found on exploration times in T1 (e1, $F_{3,92}$ = 4.99; P = 0.003) and T2 (e2, $F_{3,92}$ = 12.90; P = 0.000). Post hoc analyses revealed a tendency towards significantly higher exploration times in T1 in the saline condition when compared to the 0.03 mg/kg (P = 0.098) and 0.1 mg/kg (P = 0.076) scopolamine condition. In T2, post hoc analyses revealed significantly higher exploration times in the saline conditions when compared to all three scopolamine conditions (e2: saline compared to 0.03 mg/kg (P = 0.000), 0.1 mg/kg (P = 0.000) and 0.3 mg/kg (P = 0.001) scopolamine). Within this strain there was also an effect of the d2 index ($F_{3,92}$ = 8.74; P = 0.000). Post hoc analyses revealed that the d2 index was lower in every scopolamine condition compared to the saline condition (d2: saline compared to 0.03 mg/kg (P = 0.042), 0.1 mg/kg (P = 0.000) and 0.3 mg/kg (P = 0.000) scopolamine). One sample t-statistics only showed significant differences from zero for the d2 index of the saline condition (t_{23} = 7.63; P = 0.000). For all three scopolamine conditions (0.03, 0.1, and 0.3 mg/kg), the d2 indices did not significantly deviate from zero (t_{23} = 1.68; P = 0.106, t_{23} = -0.08; P = 0.938, and t_{23} = -0.56; P = 0.584, respectively) (see Figure 4B).

Within the Long-Evans rat strain, an effect was found of drug condition on exploration times in T1 (e1, $F_{2,67}$ = 4.22; P = 0.019) but not in T2 (e2, $F_{2,67}$ = 0.95; P = 0.392). Post hoc analyses revealed a significantly higher exploration time in T1 in the saline condition when compared to the 0.03 mg/kg scopolamine condition (P = 0.028). There did not appear to be an effect of scopolamine on the d2 indices in the Long-Evans rat strain ($F_{2,67}$ = 1.04; P = 0.361). In contrast, this strain showed significant object recognition, as indicated by one sample t-statistics, in the saline condition (t_{22} = 4.33; P = 0.000) and in the 0.03 mg/kg scopolamine condition (t_{23} = 3.81; P = 0.001), but not in the 0.1 mg/kg scopolamine condition (t_{22} = 1.67; P = 0.109) (see Figure 4D).

In the Hooded Lister rat strain, no differences were found between drug conditions on both the level of exploration in T1 (e1, $F_{2,56}$ = 0.69; P = 0.505) and

T2 (e2, $F_{2,56}$ = 0.13; P = 0.880). Furthermore, no effect of scopolamine on the d2 indices was found ($F_{2,56}$ = 2.37; P = 0.103). One sample t-statistics showed significant differences from zero for the d2 indices of the saline condition (t_{19} = 6.32; P = 0.000), and the 0.1 mg/kg scopolamine condition (t_{19} = 2.38; P = 0.028), but not of the 0.3 mg/kg scopolamine condition (t_{18} = 0.74; P = 0.466) (see Figure 4F).

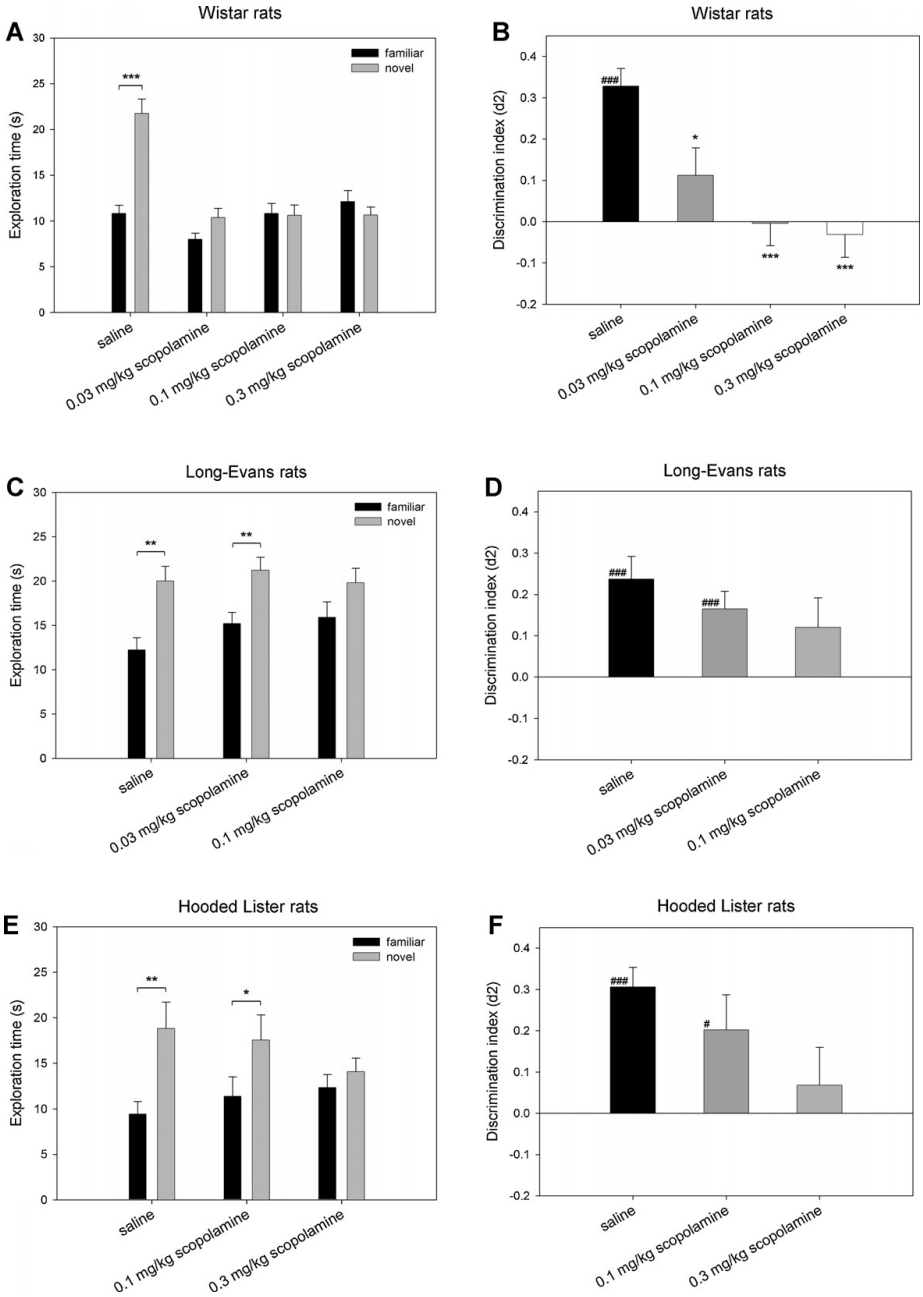

A
Wistar rats
Exploration time (s)
familiar
novel
***
saline
0.03 mg/kg scopolamine
0.1 mg/kg scopolamine
0.3 mg/kg scopolamine
B
Wistar rats
Discrimination index (d2)
###
*
***
***
saline
0.03 mg/kg scopolamine
0.1 mg/kg scopolamine
0.3 mg/kg scopolamine
C
Long-Evans rats
Exploration time (s)
familiar
novel
**
**
saline
0.03 mg/kg scopolamine
0.1 mg/kg scopolamine
D
Long-Evans rats
Discrimination index (d2)
###
###
saline
0.03 mg/kg scopolamine
0.1 mg/kg scopolamine
E
Hooded Lister rats
Exploration time (s)
familiar
novel
**
*
saline
0.1 mg/kg scopolamine
0.3 mg/kg scopolamine
F
Hooded Lister rats
Discrimination index (d2)
###
#
saline
0.1 mg/kg scopolamine
0.3 mg/kg scopolamine

Figure 4. Cognition impairing effects of scopolamine in different strains of rats. Dose-response curves of scopolamine (administration i.p., 30 min before T1) including the reference (saline) condition for the three rat strains studied in the ORT using a 1 h retention interval. n = 19-24 observations per experimental condition. **A, C & E)** Exploration times of Wistar, Long-Evans, and Hooded Lister rats after different administrated doses of scopolamine (or saline), for the familiar and novel object during T2 (mean + SEM). Significant differences in exploration times are indicated with asterisks (Paired-samples *t*-tests, *: P < 0.05; **: P < 0.01; ***: P < 0.001). **B, D & F)** Discrimination indices (d2) of Wistar, Long-Evans, and Hooded Lister rats after different administrated doses of scopolamine (or saline) (mean + SEM). Significant differences within experimental groups are indicated with asterisks (Bonferroni *t*-tests, *: P < 0.05; ***: P < 0.001). A significant difference from zero (i.e. chance level) is indicated with hashes (One sample *t*-tests, #: P < 0.05; ###: P < 0.001).

Intervening variables in object recognition testing

Object recognition in social and single housed rats

There was no effect of housing condition on exploration times in T1 (e1) or T2 (e2), (e1, $F_{1,45}$ = 1.28; P = 0.264, e2, $F_{1,45}$ = 2.58; P = 0.115). The times spent exploring both identical objects in T1 (e1) were (mean + SEM): Social housing, 25.8 sec (2.0); Single housing, 22.4 sec (1.9). The times exploring the familiar and novel objects during T2 are graphically presented in Figure 5A.

The effect of housing condition on the d2 index failed to reach significance ($F_{1,45}$ = 1.98; P = 0.166). One-sample *t*-statistics revealed that the d2 indices of both groups were significantly different from zero (social housing, t_{23} = 3.35; P = 0.003, single housing, t_{22} = 3.79; P = 0.001), indicating that recognition of the familiar object was present in both experimental groups (see Figure 5B).

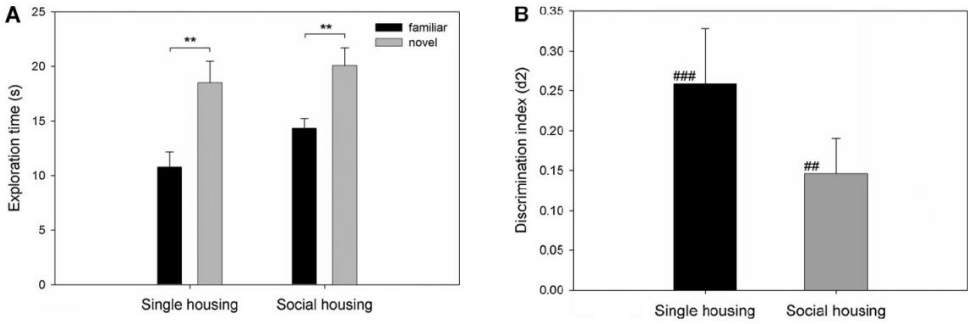


Figure 5. Object recognition in single and social housed rats. A) Exploration times of single and social housed rats for the familiar and novel object during T2 (mean + SEM) after a 1 h retention interval. The exploration times of the familiar and novel objects differed significantly in both experimental groups and are indicated with asterisks (Paired-samples *t*-tests, **: P < 0.01). **B)**

Discrimination indices (d2) of single and social housed rats (mean + SEM). No significant effect of experimental group was found. A significant difference from zero (i.e. chance level) is indicated with hashes (One sample *t*-tests, ##: P < 0.01; ###: P < 0.001). n = 23-24 observations per experimental condition.

The effects of sex and estrous cycle on object recognition in rats

There was no effect of sex and estrous cycle phase on exploration times in T1 (e1) or T2 (e2), (e1, $F_{2,32}$ = 0.54; P = 0.591, e2, $F_{2,32}$ = 1.53; P = 0.231). The times spent exploring both identical objects in T1 (e1) were (mean + SEM): Male, 28.7 sec (1.2); Female pro/es, 32.1 sec (1.7); Female met/di, 28.7 sec (1.6). The times exploring the familiar and novel objects during T2 are graphically presented in Figure 6A.

There was no effect of sex on the d2 index ($F_{1,33}$= 0.04; P = 0.846). However, there was an effect of estrous cycle phase on the d2 index ($F_{2,32}$ = 5.85; P = 0.007). Post hoc analyses revealed that the d2 index was significantly lower in females met/di compared to females pro/es (P = 0.005). One-sample *t*-statistics revealed that the d2 indices of all three groups were significantly different from zero (male, t_{11} = 6.41; P = 0.000, female pro/es, t_{10} = 10.83; P = 0.000, female met/di, t_{11} = 4.30; P = 0.001), indicating that recognition of the familiar object was present in all three experimental groups (see Figure 6B).

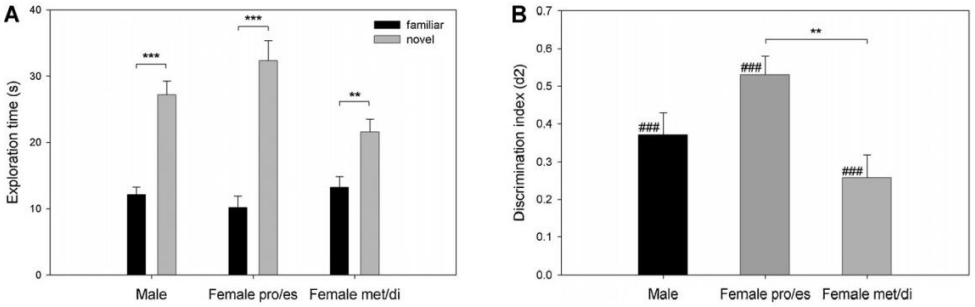


Figure 6. The effects of sex and estrous cycle on object recognition in rats. A) Exploration times of different experimental groups (male, female in pro-estrus/estrus and female in met-estrus/di-estrus) for the familiar and novel object during T2 (mean + SEM) using a 1 h retention interval. The exploration times of the familiar and novel objects differed significantly in all experimental groups and are indicated with asterisks (Paired-samples *t*-tests, **: P < 0.01; ***: P < 0.001). **B)** Discrimination indices (d2) of different experimental groups (mean + SEM). Significant differences between experimental groups were found. Female rats in pro-estrus/estrus showed higher object discrimination performance than female rats in met-estrus/di-estrus, as indicated with asterisks (Bonferroni *t*-tests, **: P < 0.01). A significant difference from zero (i.e. chance level) is indicated with hashes (One sample *t*-tests, ###: P < 0.001). n = 11-12 animals per experimental condition.

Conclusions & Discussion

Species and strain generality in object recognition testing

Object recognition in golden hamsters (Mesocricetus auratus)

The ORT performance of golden (Syrian) hamsters was assessed in order to investigate how this rodent species performs in this task. The main objective of this experiment was to extend the knowledge regarding the generality of object recognition, i.e. whether object recognition is a common ability among rodent species. Object exploration times were sufficient (> 20 sec) to enable reliable conclusions about their recognition ability (Şik et al., 2003; Akkerman et al., 2012). Inter-trial intervals of 10 min, 1 h, and 4 h resulted in significant object recognition and although discrimination performance appeared to decrease with increasing retention interval, this reduction did not reach significance. At a retention interval of 24 h, the recognition performance did not deviate from chance level, i.e. the animals did not remember the objects. Since no ORT objects familiarization sessions were performed for this study, the results of the first experiments show object recognition with absolute novel objects, which is usually higher than with familiarized objects (Akkerman et al., 2012). This implicates that the forgetting curve of the hamsters could descend when the animals are familiarized with the objects. Since the order of delay testing was not counterbalanced in this experiment, the effects seen could be partly related to re-testing effects, i.e. that the hamsters have lower exploration after multiple trials due to a lower interest. Since the analysis of variance (ANOVA) did not indicate that the duration of the retention interval affected exploration times for the familiar object in T2, this seems however unlikely. Furthermore, one should take into account the relatively small sample size. The forgetting curve appears to be similar to the ones for mice and rats (e.g. Bertaina-Anglade et al., 2006), and hence it seems that golden (Syrian) hamsters can be used reliably in the ORT. Choosing golden hamsters over rats or mice in memory research could prove to be useful in studies investigating circadian rhythms (e.g. Cain and Ralph, 2009) in this respect. The ORT may prove to be a valuable tool in this line of research.

Object recognition in different strains of mice

In line with the previous experiment, three mouse strains; OF1, NMRI, and SJL mice, were tested for their baseline ORT performance. OF1 and NMRI mice showed significant object recognition after a 1 h retention interval. In contrast, the SJL mice showed no retention after this interval. In this study the mice were adapted to the arena without any objects but ORT objects familiarization

sessions were not performed. Since the animals were only tested twice and there were four objects available, in each session a different set of objects could be shown to the mice. This way only absolute novel objects were used and baseline performance was assessed. This implicates that the ORT performance of the mice could have been slightly lower when the animals had been previously familiarized with the objects (as seen with rats: Akkerman et al., 2012).

Exploration times were very low in the SJL strain, i.e. 5 sec for both objects. With exploration times this low, a possible floor effect cannot be excluded. When the animals do not show enough object exploration in the given fixed trial duration (in this case 3 min), recognizing the objects in the subsequent trial may prove to be too difficult. On the other hand, very low exploration times may decrease the experimenters' ability to accurately score the exploration times of the mice, especially if the separate explorations last only fractions of a second. Şik and co-workers (2003) recommend using mouse strains that explore at least 10 sec during T2 in order to avoid erroneous conclusions (Şik, et al., 2003). Therefore, the SJL strain is unsuited for experiments using the ORT in the way described here, i.e. with a 3 min fixed trial duration and a 1 h retention interval.

The adult male SJL mice were very aggressive, corroborating observations by Balogh and Wehner (2003), and were consequently housed individually. Unfortunately, a few weeks of isolation may have further increased the level of aggression of the SJL mice (Lumley et al., 2004). The heightened level of aggression of these mice, however, did not obstruct the assessment of the ORT, because this task requires only minimal handling of the animals. Furthermore, from the data gathered from the social and single housed rats, it could be expected that single housed animals have better ORT performance compared to social housed animals. The finding that SJL mice have a lower ORT performance compared to the other two strains, despite the single housing conditions, would suggest this strain to be less suited for ORT experiments using a retention interval of 1 h or longer with a 3 min fixed trial duration. However, it deserves recommendation to compare single and social housed mice for ORT performance in order to be certain that these effects translate to other rodent species. In addition, it has been reported that SJL mice suffer from retinal degeneration (e.g. Crawley et al., 1997). Rodents may, however, be able to solve object discrimination tasks via tactile cues (Cybulska-Klosowicz and Kossut, 2001; Harvey, Bermejo, and Zeigler, 2001; Moreno et al., 2010). A prerequisite would be that the contact time with the object(s) is long enough to acquire a tactile representation of the object(s). This obviously was not the case for the SJL mice, since their total exploration times were very short, and hence it appears the SJL

mice did not use an alternative route of information processing here. These findings underline that a minimal total exploration time should be taken into account when assessing the performance of mouse strains in the ORT.

Memory impairing effects of scopolamine in different strains of rats

The aim of this experiment was to separately assess the ORT performance of three different rat strains after administration (i.p.) of scopolamine. In all three strains a dose dependent relationship of scopolamine was found. However, there were differences between the three strains with regard to the required doses of scopolamine needed to induce an object recognition deficit. The lowest doses of scopolamine to induce the targeted object recognition deficit (i.e. d2 index not significantly different from zero), were 0.03, 0.1, and 0.3 mg/kg for Wistar, Long-Evans and Hooded Lister rats, respectively. A one-way ANOVA indicated that the baseline performance of the rats, i.e. the saline/vehicle condition, were similar. Therefore, all three rat strains are suited for experiments using the ORT in the way described here, i.e. with a 3 min fixed trial duration and a 1 h retention interval. Since not every rat strain received all scopolamine conditions, between-group comparisons could not adequately be performed.

Significant differences were found between drug conditions for the exploration times during both T1 and T2 within the Wistar rats, and for the exploration times in T1 within the Long-Evans rats. In both strains it concerned a difference between the saline condition and one or more scopolamine conditions (saline > scopolamine). Figure 4A clearly shows higher exploration times in T2 for the Wistar rats in the saline condition than in each of the scopolamine conditions. It is assumed that even the lowest dose of scopolamine (0.03 mg/kg) caused some sedation in these Wistar rats, which resulted in lower exploration times when compared to the saline condition. For the Long-Evans rats, a difference was only found between the saline and the 0.03 mg/kg scopolamine conditions in T1. Because this difference was not found with the 0.1 mg/kg scopolamine condition in T1, this is not assumed to be an effect of sedation, but rather an incidental effect.

Earlier research led to inconsistent results concerning the effect of cholinergic depletion/blockade on cognition in different rat strains. In a study that directly compared the effects of scopolamine in Long-Evans and Flinders rats, no obvious cognitive differences were discovered (Bushnell et al., 1995). In a comparative study of spatial radial maze performance, three strains of male rats (Fischer 344, Sprague-Dawley and Wistar rats) were used to assess the cognition-impairing effect of scopolamine. The effect of scopolamine differed among strains, with

the Wistar rat being the most affected by the administration of scopolamine (Higashida and Ogawa, 1987). In another study, which used three separate learning and memory paradigms, differences were found in performance between four rat strains (Long-Evans, Sprague-Dawley, Wistar and Tryoan maze dull (S3) rats) following treatment with the cholinergic-depleting agent hemicholinium-3 (HC3) (Andrews et al., 1995). Moreover, in yet another study investigating rat strains, between-strain differences in response to galanin (an inhibitory modulator of cholinergic transmission) in the Morris water maze (MWM) were found. Galanin impaired acquisition of the hidden platform location in the MWM in Sprague-Dawley and Wistar rats, but not in Long-Evans rats (Gleason et al., 1999). Taken together, these studies indicate that different strains of rats show distinct task-dependent learning abilities and susceptibilities to cholinergic depletion. Furthermore, rat strain differences in the cholinergic system itself have been reported as well (e.g. Michalek et al., 1989).

Another possible explanation for these phenomena and the results presented in the present study could be that the different rat strains used include albino and pigmented animals. The Wistar rats are an albino strain and the Long-Evans and Hooded Lister rats are pigmented. It has been put forward that differences found in pigmented and albino strains could result from the interaction of different drugs with (neuro)melanin in pigment granules (Sisson et al., 1991), of which pigmented animals have more than albino animals (e.g. Conlee et al., 1989). Considering much toxicological testing and preclinical drug screening is still performed on albino animals, concern has been raised by drug regulatory agencies and the pharmaceutical industry regarding the ability of different drugs to interact with (neuro)melanin (Zane et al., 1990).

A possible confounder in these types of studies is the difference in visual acuity between pigmented and albino strains. Prusky and co-workers (2002) measured the visual function of several pigmented and albino strains of laboratory rats. Albino strains possessed approximately half the visual acuity of the pigmented strains (Prusky et al., 2002). Considering that the Wistar rats were visually impaired when compared to the Long-Evans and Hooded Lister rats, and that the ORT is at least partly dependent upon visual cues (Mumby, 2001), this could have influenced the results.

Future studies in which several albino rat strains are directly compared to pigmented rat strains are recommended to reliably draw conclusions about this subject. Taken together, investigators should keep strain differences in mind when using a pharmacological induced memory deficit model.

Intervening variables in object recognition testing

Object recognition in social and single housed rats

The effects of single and social housing (groups of three) conditions on ORT performance in male Wistar rats were investigated. Both groups showed significant object recognition as indicated by the one-sample *t*-statistics. Yet the single housed rats showed an absolute higher discrimination index (d2) than the social housed rats (0.26 and 0.15, respectively), but this difference failed to reach significance. Nevertheless, these results indicate that investigators should consider housing conditions as an intervening variable in the ORT.

The lower absolute discrimination performance in the socially housed group could have been caused by too much interference between T1 and T2. Because the rats are placed back in their home cages during the inter-trail retention interval, they are exposed to the other rats, which might be an interfering factor. Beck and Luine (2002) found that housing condition was a major factor in determining the performance of male Sprague-Dawley rats in the ORT. The pairwise housing of the male rats led to inferior performance when compared to single housing (Beck and Luine, 2002).

The effects of sex and estrous cycle on object recognition in rats

Male and female Wistar rats were tested to assess the effects of sex and estrous cycle phase on ORT performance. After a 1 h interval, all experimental groups showed significant object recognition. No overall sex effect was found. In contrast, females in pro-estrus/estrus had significantly better object recognition performance compared to females in met-estrus/di-estrus, indicating an effect of estrous cycle phase.

Previous studies also found significant object recognition memory after a 1 h retention interval in male (Lieben et al., 2005; Lieben et al., 2006; Rutten et al., 2007) and female Wistar rats (Jans et al., 2007). In contrast to the present study, a sex effect in favour of female rats has been reported previously (for Wistar rats: (Ghi et al., 1999); for Hooded Lister rats: (Sutcliffe et al., 2007)). However, in these studies longer lasting memory retrieval in females compared to males was found when the retention interval was increased, an aspect that was not examined in the present study. The finding of an estrous cycle phase effect on ORT performance could confound studies using female subjects. Usually, female rodents and other mammals living in groups synchronize their estrous cycles (McClintock, 1984), making it unlikely that the females in one experiment are evenly distributed across all estrous cycle phases.

The female rats were subdivided in groups based on either higher (pro/es) or lower (met/di) levels of oestradiol and progesterone (Contreras et al., 2000). It has been shown that oestradiol and progesterone can improve memory acquisition (Gibbs, 2000) and memory retention (Sandstrom and Williams, 2001) in female rats. It has been suggested that these changes may be the result of hormone-induced increases in hippocampal connectivity. Indeed, it has been demonstrated that low levels of oestradiol correlate with a lower hippocampal synaptic density, while higher levels of oestradiol correlate with higher hippocampal synaptic density (Woolley and McEwen, 1992). This may, to some extent, explain our results. Different performances of female rats in different estrous cycle phases in spatial and non-spatial memory tasks have been found before, but often other subdivisions than the ones herein described where used (e.g. Warren and Juraska, 1997).

The females may have been exposed to higher levels of stress than the males, because of the vaginal smears that were taken in this study. However, given the smears were taken at least three hours preceding testing, and the clear effect of estrous cycle phase, we do not believe stress effects to have confounded ORT performance of the female rats.

Considerations for the object recognition task

An advantage of the ORT is that it is applicable in most commonly used laboratory rodents including mouse, rat, and hamster, provided suitable strains are selected (Şik et al., 2003). An entire experiment can be performed in a relatively short period of time, i.e. the ORT has a relatively high throughput and requires only little training/familiarization. Furthermore, the ORT is a two-trial paradigm, based on the animals' natural tendency to explore novelty. This opens the possibility to assess the effects of putative cognition-modulating compounds on the acquisition, consolidation, and retrieval processes of memory (e.g. (Lamirault and Simon, 2001; Prickaerts et al., 2005); see also Figure 7, top picture), and to further analyse the precise dynamics of the consolidation process. For example, a distinction can be made between early and late consolidation (e.g. Rutten et al., 2006). It should be mentioned that the pharmacokinetics of a test compound must always be kept in mind, e.g. the administration of a compound before T1, administrated to target the acquisition process, can still influence consolidation providing that the dose and half-life of a compound are high enough (see Figure 7, bottom picture). Another advantage of this paradigm is that each animal can be tested repeatedly under the same, or modified experimental conditions (e.g. different retention intervals or different

doses of a test compound). This repeated testing allows for within-subjects designs which reduce error and hence increase the power of a study. Consequently, this allows for reduction in the number of animals needed in an experiment (Akkerman et al., 2012).

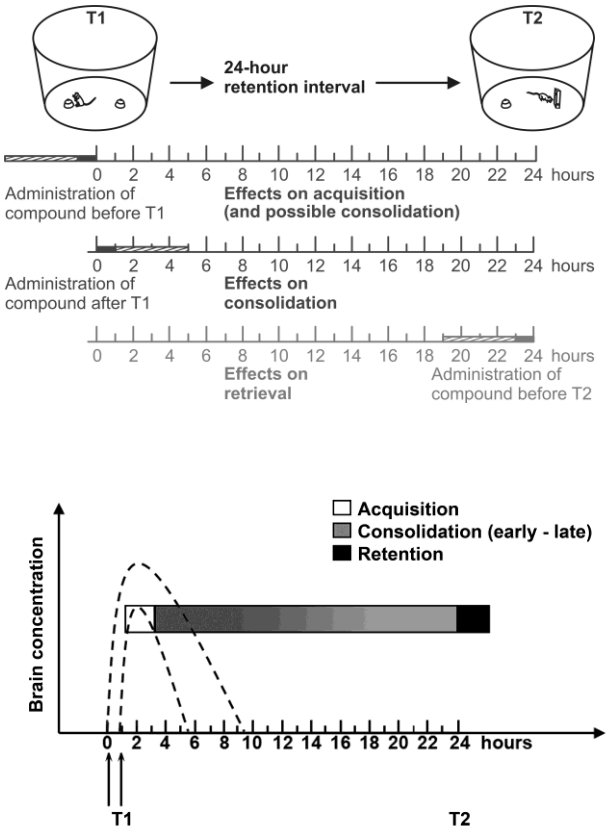


Figure 7. Considerations for the object recognition task. *Upper panel):* Schematic representation of administration schedules for assessing effects of putative memory enhancers on acquisition, consolidation and/or retrieval processes in the ORT with a 24 h retention interval. The administration time points are depicted as filled and hatched horizontal bars on the timelines. *Lower panel*): Schematic representation showing that a compound given before T1 (time point: 0 h), to target the acquisition process, can affect consolidation given the compounds' brain concentration is high enough. Vertical arrows underneath the timeline indicate administration of compounds. Dotted lines indicate the brain concentration of the administrated compound. The durations of acquisition, consolidation and retention processes are estimated.

One should keep in mind that ORT outcomes do not have to solely reflect memory processes. Other cognitive processes needed for memory formation, like for example attention processes, could probably also contribute. The involvement of acetylcholine and cholinergic drugs in attention processes instead of specific memory processes has been put forward earlier (Blokland, 1995). This shows that, in behavioral testing, it is rather difficult to see these cognitive processes as separate, since cholinergic drugs can enhance ORT performance (e.g. Prickaerts et al., 2005). Another important issue concerning

the ORT, as also became clear from the studies herein described, is that it is essential to always consider the effects of species, strain, housing, and estrous cycle when the ORT performance is assessed. These effects can obscure interpretation of for example drug effects. Furthermore, treatments/drugs that reduce locomotor behavior may confound the measures of object discrimination. A solution to this limitation is the use of the discrimination index d2, which is independent of exploratory behavior (Akkerman et al., 2012). It should be stressed that a minimal exploration time (>10 sec) is recommended in order to achieve reliable discrimination indices (Şik et al., 2003; Akkerman et al., 2012). An alternative is to leave an animal in the apparatus until a certain amount of exploration time is reached. An advantage of this approach is that this way all animals have the same amount of object exposure. Furthermore, the amount of time an animal explores could be controlled this way. A drawback of this approach is that the planning of studies becomes overly complex.

The ORT accounts for three kinds of sensory information available to an animal in this task; visual, tactile, and olfactory information (Mumby, 2001). The olfactory information is controlled for by means of thoroughly cleaning the objects after each trial. That leaves visual and tactile information to which the animals have to respond. Many mouse and rat strains, in particular albino strains, suffer from retinal degenerations which may influence the performance in a usual ORT. Also, the aging process may compromise vision (e.g. Militante and Lombardini, 2004) and limit the use of aged animals. Moreno and co-workers showed that a 'tactile' version of the ORT (objects with the same shape, size, and colour but with different textures), allows recognition testing in visually impaired albino subjects (Wistar rats) (Moreno et al., 2010). Interestingly, it has also been shown that pigmented Long-Evans rats are able to have object recognition in a pure visual task in which the rats were prevented from having physical contact with the objects (Winters and Reid, 2010).

At present the ORT has a strong position as a primary behavioral screen for testing putative memory enhancing compounds. The paradigm is suited to assess memory acquisition, consolidation, and retrieval processes while avoiding reinforcement or rule learning. When keeping the herein mentioned considerations of strain, species, housing conditions, and estrous cycle in mind, the reliability of the results is warranted and the ORT continues to be a valuable tool in behavioral and pharmacological research.

Acknowledgements
The authors like to thank Dorota Chodor and Eva van Donkelaar for the expert technical assistance.

References

Akkerman, S., Blokland, A., Reneerkens, O. H. A., van Goethem, N. P., Bollen, E., Gijselaers, J., et al. (2012). Object recognition testing: Methodological considerations on exploration and discrimination measures. *Behavioural Brain Research*, 232(2), 335-347.

Andrews, J. S., Jansen, J. H. M., Linders, S., Princen, A., and Broekkamp, C. L. E. (1995). Performance of four different rat strains in the autoshaping, two-object discrimination, and swim maze tests of learning and memory. *Physiology & Behavior, 57*(4), 785-790.

Astur, R. S., Klein, R. L., Mumby, D. G., Protz, D. K., Sutherland, R. J., and Martin, G. M. (2002). A role for olfaction in object recognition by normal and hippocampal-damaged rats. *Neurobiology of Learning and Memory, 78*(1), 186-191.

Balogh, S. A., and Wehner, J. M. (2003). Inbred mouse strain differences in the establishment of long-term fear memory. *Behavioural Brain Research, 140*(1-2), 97-106.

Beck, K. D., and Luine, V. N. (2002). Sex differences in behavioral and neurochemical profiles after chronic stress: Role of housing conditions. *Physiology & Behavior, 75*(5), 661-673.

Becker, J. B., Arnold, A. P., Berkley, K. J., Blaustein, J. D., Eckel, L. A., Hampson, E., et al. (2005). Strategies and methods for research on sex differences in brain and behavior. *Endocrinology, 146*(4), 1650-1673.

Bertaina-Anglade, V., Enjuanes, E., Morillon, D., and Drieu la Rochelle, C. (2006). The object recognition task in rats and mice: a simple and rapid model in safety pharmacology to detect amnesic properties of a new chemical entity. *Journal of Pharmacological and Toxicological Methods, 54*(2), 99-105.

Blokland, A. (1995). Acetylcholine: a neurotransmitter for learning and memory? *Brain Research Reviews, 21*(3), 285-300.

Blokland, A., Rutten, K., and Prickaerts, J. (2006). Analysis of spatial orientation strategies of male and female Wistar rats in a Morris water escape task. *Behavioural Brain Research, 171*(2), 216-224.

Bour, A., Little, S., Dodart, J. C., Kelche, C., and Mathis, C. (2004). A secreted form of the [beta]-amyloid precursor protein (sAPP695) improves spatial recognition memory in OF1 mice. *Neurobiology of Learning and Memory, 81*(1), 27-38.

Bredy, T. W., Lee, A. W., Meaney, M. J., and Brown, R. E. (2004). Effect of neonatal handling and paternal care on offspring cognitive development in the monogamous California mouse (Peromyscus californicus). *Hormones and Behavior, 46*(1), 30-38.

Bushnell, P. J., Levin, E. D., and Overstreet, D. H. (1995). Spatial working and reference memory in rats bred for autonomic sensitivity to cholinergic stimulation: acquisition, accuracy, speed, and effects of cholinergic drugs. *Neurobiology of Learning and Memory, 63*(2), 116-132.

Cain, S. W., and Ralph, M. R. (2009). Circadian modulation of conditioned place avoidance in hamsters does not require the suprachiasmatic nucleus. *Neurobiology of Learning and Memory, 91*(1), 81-84.

Collins, D. W., and Kimura, D. (1997). A large sex difference on a two-dimensional mental rotation task. *Behavioral Neuroscience, 111*(4), 845-849.

Conlee, J. W., Gill, S. S., McCandless, P. T., and Creel, D. J. (1989). Differential susceptibility to gentamicin ototoxicity between albino and pigmented guinea pigs. *Hearing Research, 41*(1), 43-51.

Contreras, C. M., Molina, M., Saavedra, M., and Martínez-Mota, L. (2000). Lateral septal neuronal firing rate increases during proestrus-estrus in the rat. *Physiology & Behavior, 68*(3), 279-284.

Crawley, J. N., Belknap, J. K., Collins, A., Crabbe, J. C., Frankel, W., Henderson, N., et al. (1997). Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology, 132*(2), 107-124.

Cybulska-Klosowicz, A., and Kossut, M. (2001). Mice can learn roughness discrimination with vibrissae in a jump stand apparatus. *Acta Neurobiologiae Experimentalis, 61*(1), 73-76.

Delgado, A. R., and Prieto, G. (1996). Sex differences in visuospatial ability: do performance factors play such an important role? *Memory & Cognition, 24*(4), 504-510.

Dodart, J. C., Mathis, C., and Ungerer, A. (1997). Scopolamine-induced deficits in a two-trial object recognition task in mice. *Neuroreport, 8*(5), 1173-1178.

Ennaceur, A., Cavoy, A., Costa, J. C., and Delacour, J. (1989). A new one-trial test for neurobiological studies of memory in rats. II: Effects of piracetam and pramiracetam. *Behavioural Brain Research, 33*(2), 197-207.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*(1), 47-59.

Ennaceur, A., and Meliani, K. (1992b). Effects of physostigmine and scopolamine on rats' performances in object-recognition and radial-maze tests. *Psychopharmacology, 109*(3), 321-330.

Ennaceur, A., Michalikova, S., Bradford, A., and Ahmed, S. (2005). Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. *Behavioural Brain Research, 159*(2), 247-266.

Ghi, P., Orsetti, M., Gamalero, S. R., and Ferretti, C. (1999). Sex differences in memory performance in the object recognition test. Possible role of histamine receptors. *Pharmacology Biochemistry and Behavior, 64*(4), 761-766.

Gibbs, R. B. (2000). Long-term treatment with estrogen and progesterone enhances acquisition of a spatial memory task by ovariectomized aged rats. *Neurobiology of Aging, 21*(1), 107-116.

Gleason, T. C., Dreiling, J. L., and Crawley, J. N. (1999). Rat strain differences in response to galanin on the Morris water task. *Neuropeptides, 33*(4), 265-270.

Griebel, G., Belzung, C., Perrault, G., and Sanger, D. J. (2000). Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. *Psychopharmacology, 148*(2), 164-170.

Harvey, M. A., Bermejo, R., and Zeigler, H. P. (2001). Discriminative whisking in the head-fixed rat: optoelectronic monitoring during tactile detection and discrimination tasks. *Somatosensory & Motor Research, 18*(3), 211-222.

Higashida, A., and Ogawa, N. (1987). Differences in the acquisition process and the effect of scopolamine on radial maze performance in three strains of rats. *Pharmacology Biochemistry and Behavior, 27*(3), 483-489.

Jans, L. A. W., Lieben, C. K. J., and Blokland, A. (2007). Influence of sex and estrous cycle on the effects of acute tryptophan depletion induced by a gelatin-based mixture in adult Wistar rats. *Neuroscience, 147*(2), 304-317.

Jonasson, Z. (2005). Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. *Neuroscience & Biobehavioral Reviews, 28*(8), 811-825.

Kathmann, M., Weber, B., and Schlicker, E. (2001). Cannabinoid CB 1 receptor-mediated inhibition of acetylcholine release in the brain of NMRI, CD-1 and C57BL/6J mice. *Naunyn-Schmiedeberg's Archives of Pharmacology, 363*(1), 50-56.

Lamirault, L., and Simon, H. (2001). Enhancement of place and object recognition memory in young adult and old rats by RS 67333, a partial agonist of 5-HT4 receptors. *Neuropharmacology, 41*(7), 844-853.

Lieben, C. K., Blokland, A., Şik, A., Sung, E., van Nieuwenhuizen, P., and Schreiber, R. (2005). The selective 5-HT6 receptor antagonist Ro4368554 restores memory performance in cholinergic and serotonergic models of memory deficiency in the rat. *Neuropsychopharmacology, 30*(12), 2169-2179.

Lieben, C. K. J., Steinbusch, H. W. M., and Blokland, A. (2006). 5, 7-DHT lesion of the dorsal raphe nuclei impairs object recognition but not affective behavior and corticosterone response to stressor in the rat. *Behavioural Brain Research, 168*(2), 197-207.

Lumley, L. A., Robison, C. L., Slusher, B. S., Wozniak, K., Dawood, M., and Meyerhoff, J. L. (2004). Reduced isolation-induced aggressiveness in mice following NAALADase inhibition. *Psychopharmacology, 171*(4), 375-381.

McClintock, M. K. (1984). Estrous synchrony: modulation of ovarian cycle length by female pheromones. *Physiology & Behavior, 32*(5), 701-705.

Meaney, M. J., and Stewart, J. (1981). A descriptive study of social development in the rat (Rattus norvegicus). *Animal Behaviour, 29*(1), 34-45.

Messier, C. (1997). Object Recognition in Mice: Improvement of Memory by Glucose. *Neurobiology of Learning and Memory, 67*(2), 172-175.

Michalek, H., Fortuna, S., and Pintor, A. (1989). Age-related differences in brain choline acetyltransferase, cholinesterases and muscarinic receptor sites in two strains of rats. *Neurobiology of Aging, 10*(2), 143-148.

Militante, J., and Lombardini, J. B. (2004). Age-related retinal degeneration in animal models of aging: possible involvement of taurine deficiency and oxidative stress. *Neurochemical Research, 29*(1), 151-160.

Moreno, C., Vivas, O., Lamprea, N. P., Lamprea, M. R., Múnera, A., and Troncoso, J. (2010). Vibrissal paralysis unveils a preference for textural rather than positional novelty in the one-trial object recognition task in rats. *Behavioural Brain Research, 211*(2), 229-235.

Mumby, D. G. (2001). Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. *Behavioural Brain Research, 127*(1-2), 159-181.

Mumby, D. G., Glenn, M. J., Nesbitt, C., and Kyriazis, D. A. (2002). Dissociation in retrograde memory for object discriminations and object recognition in rats with perirhinal cortex damage. *Behavioural Brain Research, 132*(2), 215-226.

Norman, G., Brooks, S. P., Hennebry, G. M., Eacott, M. J., and Little, H. J. (2002). Nimodipine prevents scopolamine-induced impairments in object recognition. *Journal of Psychopharmacology, 16*(2), 153-161.

Palchykova, S., Crestani, F., Meerlo, P., and Tobler, I. (2006). Sleep deprivation and daily torpor impair object recognition in Djungarian hamsters. *Physiology & Behavior, 87*(1), 144-153.

Petrulis, A. (2009). Neural mechanisms of individual and sexual recognition in Syrian hamsters (Mesocricetus auratus). *Behavioural Brain Research, 200*(2), 260-267.

Prickaerts, J., de Vente, J., Honig, W., Steinbusch, H. W. M., and Blokland, A. (2002a). cGMP, but not cAMP, in rat hippocampus is involved in early stages of object memory consolidation. *European Journal of Pharmacology, 436*(1-2), 83-87.

Prickaerts, J., Sik, A., van der Staay, F. J., de Vente, J., and Blokland, A. (2005). Dissociable effects of acetylcholinesterase inhibitors and phosphodiesterase type 5 inhibitors on object recognition memory: acquisition versus consolidation. *Psychopharmacology, 177*(4), 381-390.

Prickaerts, J., Steinbusch, H. W. M., Smits, J. F. M., and de Vente, J. (1997). Possible role of nitric oxide-cyclic GMP pathway in object recognition memory: effects of 7-nitroindazole and zaprinast. *European Journal of Pharmacology, 337*(2-3), 125-136.

Prickaerts, J., van Staveren, W. C. G., Şik, A., Markerink-van Ittersum, M., Niewohner, U., van der Staay, F. J., et al. (2002b). Effects of two selective phosphodiesterase type 5 inhibitors, sildenafil and vardenafil, on object recognition memory and hippocampal cyclic GMP levels in the rat. *Neuroscience, 113*(2), 351-361.

Prusky, G. T., Harker, K. T., Douglas, R. M., and Whishaw, I. Q. (2002). Variation in visual acuity within pigmented, and between pigmented and albino rat strains. *Behavioural Brain Research, 136*(2), 339-348.

Rutten, K., Lieben, C., Smits, L., and Blokland, A. (2007). The PDE4 inhibitor rolipram reverses object memory impairment induced by acute tryptophan depletion in the rat. *Psychopharmacology, 192*(2), 275-282.

Rutten, K., Prickaerts, J., and Blokland, A. (2006). Rolipram reverses scopolamine-induced and time-dependent memory deficits in object recognition by different mechanisms of action. *Neurobiology of Learning and Memory, 85*(2), 132-138.

Sandstrom, N. J., and Williams, C. L. (2001). Memory retention is modulated by acute estradiol and progesterone replacement. *Behavioral Neuroscience, 115*(2), 384-393.

Şik, A., van Nieuwehuyzen, P., Prickaerts, J., and Blokland, A. (2003). Performance of different mouse strains in an object recognition task. *Behavioural Brain Research, 147*(1-2), 49-54.

Singletary, S. J., Kirsch, A. J., Watson, J., Karim, B. O., Huso, D. L., Hurn, P. D., et al. (2005). Lack of correlation of vaginal impedance measurements with hormone levels in the rat. *Contemporary Topics in Laboratory Animal Science, 44*(6), 37-42.

Sisson, D. F., Siegel, J., and Westenberg, I. S. (1991). Are the differential effects of chloral hydrate on hooded rats vs. albino rats due to pigmentation or strain differences? *Pharmacology Biochemistry and Behavior, 39*(3), 665-670.

Sutcliffe, J. S., Marshall, K. M., and Neill, J. C. (2007). Influence of gender on working and spatial memory in the novel object recognition task in the rat. *Behavioural Brain Research, 177*(1), 117-125.

Vaucher, E., Fluit, P., Chishti, M. A., Westaway, D., Mount, H. T. J., and Kar, S. (2002). Object recognition memory and cholinergic parameters in mice expressing human presenilin 1 transgenes. *Experimental Neurology, 175*(2), 398-406.

Warren, S. G., and Juraska, J. M. (1997). Spatial and nonspatial learning across the rat estrous cycle. *Behavioral Neuroscience, 111*(2), 259-266.

Winters, B. D., and Reid, J. M. (2010). A distributed cortical representation underlies crossmodal object recognition in rats. *The Journal of Neuroscience, 30*(18), 6253-6261.

Woolley, C. S., and McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *The Journal of Neuroscience, 12*(7), 2549-2554.

Zane, P. A., Brindle, S. D., Gause, D. O., O'Buck, A. J., Raghavan, P. R., and Tripp, S. L. (1990). Physicochemical factors associated with binding and retention of compounds in ocular melanin of rats: correlations using data from whole-body autoradiography and molecular modeling for multiple linear regression analyses. *Pharmaceutical Research, 7*(9), 935-941.

Chapter 4

Rodent models of cognitive disorders: Impairment, aging & dementia

Nick P. van Goethem[1], Roy Lardenoije[1], Konstantinos Kompotis, Bart P.F. Rutten, Jos Prickaerts and Harry W.M. Steinbusch

In vivo models for drug discovery (2014). Wiley-VCH Verlag, 347-364

Abstract

Cognitive dysfunction is a feature often encountered in a broad spectrum of neurological and psychiatric conditions. Animal models to study the development of these disorders are crucial to understand how cognitive deficits are often the result of neurodegeneration. In addition, since ameliorating these dysfunctions can dramatically improve the quality of life of patients, developing treatments, or 'cognition enhancers', is a major area of interest for pharmaceutical industry. A diverse range of animal models is being used to identify potential cognition enhancing drugs and such models can be based on pharmacological deficits, the naturally occurring aging process and/or introduction of transgenic constructs in rodents. In pharmacological deficit models, specific drugs are administered to animals in order to induce cognitive deficits. Of most cognitive disorders aging is the top risk factor. Aging can result in severe functional limitations at the end of the life-span. Rats and mice are useful laboratory species for studying the aging process and consequential cognitive deficits. Another way to investigate cognitive deficits in animals is by using transgenic animals. Over the past few decades, transgenic rodent models modeling specific diseases and exhibiting cognitive deficits have been generated. A representative selection of transgenic rodent models exhibiting cognitive impairments in relation to common neurodegenerative processes and diseases, are described in this chapter. Lastly, translation of these models to the clinics and their limitations and difficulties are described. Different validities, and to what extent these animal models satisfy these criteria, are explained.

Cognitive dysfunction is a feature often encountered in a broad spectrum of neurological and psychiatric conditions. The property of animal models to study the development of a disease and not just late-stage pathology, is crucial for disease models involving cognitive deficits, as such deficits are often the result of neurodegeneration. Considering the limited regenerative capacity of the brain, it is thus pivotal to treat neurodegenerative diseases as early as possible (Faigle and Song, 2013). Since ameliorating these dysfunctions can dramatically improve the quality of life of patients, developing treatments, or 'cognition enhancers', is a major area of interest for the pharmaceutical industry. Accordingly, over the last few decades certain drugs have been approved for the treatment of cognitive impairments related to specific neurological and psychiatric conditions (for a recent review see: Froestl et al., 2012). A diverse range of animal models is being used to identify potential cognition enhancing drugs and such models can be based on pharmacological deficits, the naturally occurring aging process and/or introduction of transgenic constructs in rodents. The first part of this chapter describes the most commonly used rodent pharmacological deficit models. Hereafter, animal models of aging and transgenic animal models will be described.

Pharmacological models

In pharmacological deficit models, specific drugs are administered to animals in order to induce cognitive deficits. The targets of these cognition-impairing drugs are hypothesis-based and are often directed to alter distinct neurotransmitter systems, with different disorders showing specific dysregulations or impairments.

Inhibition of energy/glucose metabolism

A variety of studies in both rodents and humans has shown that slight increases in circulating glucose concentrations exhibit beneficial effects in brain functions relating to learning and memory (Gold, 1995). Administrating glucose has been shown to facilitate rodent performance and furthermore reverses both drug- and age-related cognitive deficits. The putative mechanism of action underlying these pro-cognitive effects probably relate to altered neuronal metabolism, neuronal activity or neurotransmitter synthesis (Korol and Gold, 1998).

The most straightforward way to inhibit energy/glucose metabolism is by glucose deprivation. *In vitro* studies often use oxygen-glucose deprivation to mimic ischemic injury and subsequently study acute stroke pathology (e.g. Cho et al., 2007). *In vivo* studies which use oxygen-glucose deprivation mostly do this

via middle cerebral artery occlusion (e.g. Bederson et al., 1986). NMDA (N-methyl-D-aspartate) receptor antagonists have been shown to be neuroprotective against excitotoxicity in both *in vitro* and *in vivo* models of ischemia or neurodegeneration (Arias et al., 1999; Danysz and Parsons, 2003). Another way to inhibit energy/glucose metabolism is by treatment with the glycolytic inhibitor, 2-deoxyglucose. Whereas mostly used for glucose uptake measurement, 2-deoxyglucose has been shown to dose-dependently affect cognitive performance of rodents (Ockuly et al., 2012).

Another possible animal model for metabolic dysfunction (and/or generation of oxidative stress) is intracerebral-ventricular (i.c.v.) administration of streptozotocin (Nitsch et al., 1989; Mayer et al., 1990). Streptozotocin is a naturally occurring chemical that was originally discovered in the late 1950s and a little later identified as an antibacterial antibiotic (Vavra et al., 1959). Subsequently, it was discovered that i.c.v. administration of streptozotocin decreases the central metabolism of glucose and hence offers a useful animal/rodent model of neurodegeneration (e.g. Alzheimer's disease) (Prickaerts et al., 1995). Furthermore, i.c.v. streptozotocin administration also reduces the concentrations of different neurotransmitters, including acetylcholine (ACh) (Ding et al., 1992; Hellweg et al., 1992). As will be described in the next section, this cholinergic reduction further contributes to the use of this animal model of neurogeneration. Accordingly, middle-aged and old rats which have been treated with streptozotocin (i.c.v.) show cognitive deficits in tasks assessing learning and memory. These deficits can be reversed with specific cognition enhancing drugs (Blokland and Jolles, 1993; Prickaerts et al., 1995).

Cholinergic interventions

Cholinergic toxins

The use of pharmacological deficit models targeting the cholinergic system became popular after the cholinergic hypothesis of geriatric memory dysfunction was postulated. This hypothesis states that the age-related decline in cognition is predominately caused by a decrement of cholinergic neurotransmission (Bartus et al., 1982). Nowadays, with the exception of one NMDA receptor antagonist (see also above), all approved drugs for the treatment of cognitive dysfunction in Alzheimer's disease aim at increasing cholinergic neurotransmission. Different approaches have been used to induce cholinergic hypofunction in order to mimic Alzheimer's disease-, and age-related cognitive decline. To achieve chronic dysregulation of the cholinergic system, cholinergic toxins have been used. The exact role of ACh in cognition is not fully

understood, but ACh regulation has been associated with attention-, learning- and memory-processes (Blokland, 1995).

Many of the early rat studies made use of excitotoxic lesions by means of central administrations of ibotenic acid or quisqualic acid. The excitotoxic lesions (especially with ibotenic acid) of cholinergic neurons revealed a vast range of cognitive impairments (Peternel et al., 1988; Steckler et al., 1993). However, a fundamental problem with this approach was the lack of a specific cholinergic toxin, introducing the possibility that such impairments may be due to damage to non-cholinergic neurons. A more selective way to destruct cholinergic cells can be accomplished by locally injecting 192 IgG-saporin. 192 IgG-saporin is an antineuronal immunotoxin which consists of a monoclonal antibody (192 IgG) to the nerve growth factor (NGF) receptor that has been armed with saporin, a ribosome-inactivating protein (Wiley et al., 1991; Wenk et al., 1994). Injections of this 192 IgG-saporin complex produce long-lasting depletions in cholinergic markers throughout the forebrain of rats (Book et al., 1992). 192 IgG-saporin administration has been used to induce cognitive impairments in rodents to investigate the role of the cholinergic system in particular brain structures (e.g. Walsh et al., 1996; Lehmann et al., 2003).

Cholinergic antagonists

Induction of more transient or acute disruption of the cholinergic system can be induced with cholinergic antagonists. ACh has two types of receptors; the metabotropic muscarinic receptors (5 subtypes in CNS), and the ionotropic nicotinic receptors (2 major subtypes in CNS). There are specific antagonists for each ACh receptor type. A further division can be made between selective and non-selective cholinergic antagonists. This applies to the selectivity/affinity of an antagonist to the isoforms of ACh receptor (sub)types.

The most widely used non-selective competitive cholinergic antagonists are the tropan alkaloids scopolamine hydrobromide and atropine. The non-selective muscarinic antagonist scopolamine is probably the most often used cognition-deficit inducing drug in (preclinical) rodent research. Since scopolamine induces amnesia that is caused by a blockade of cholinergic signaling, this drug is used to model cognitive deficits associated with aging and dementia (Klinkenberg and Blokland, 2010). In preclinical testing, scopolamine is often co-administered with putative cognition enhancing drugs in order to test whether a new drug is effective in reversing a scopolamine-induced cognitive deficit (e.g. Prickaerts et al., 2012). The rationale is that if a new experimental drug can reverse such a

deficit, it might also improve cognitive function in healthy participants or people diagnosed with a neuropsychiatric disorder (Klinkenberg and Blokland, 2010).

Since scopolamine is a non-selective muscarinic antagonist, efforts have been made to promote the use of more selective muscarinic antagonists. Since muscarinic receptors are both centrally and peripherally present, it would be more 'clean' to use a more centrally selective muscarinic antagonist. Of the 5 known muscarinic receptors (M1-M5), M1 might be a promising target since this receptor is predominantly located in the cortex and the hippocampus, brain regions known to be important for attention, learning and memory. Peripheral presence of the M1 receptor is relatively limited (Caulfield, 1993). The selective muscarinic M1 receptor antagonist biperiden is therefore an interesting drug candidate to more selectively induce cognitive, in particular memory, deficits in rodent models (Klinkenberg and Blokland, 2011).

The other class of cholinergic receptors is the class of ionotropic nicotinic receptors (nAChRs). These receptors belong to a family of ligand gated ion channel receptors which include type 3 serotonin (5-HT_3), $GABA_A$, and strychnine-sensitive glycine receptors. nAChRs in the brain are composed out of five subunits, which can either be α subunits (9 identified subunits: α2-α10) or β subunits (3 identified subunits: β2-β4). These subunits can combine to result in different isoforms. In the CNS the heteropentameric α4β2 and the homopentameric α7 nAChRs comprise >90% of the nAChR subtypes (Toyohara and Hashimoto, 2010). Since nAChRs have been shown to be involved in learning and memory (Prickaerts et al., 2012) and postmortem research shows that nAChR densities are markedly decreased in the brains of both patients with Alzheimer's disease and schizophrenia, the pharmaceutical industry has been developing different nAChR agonists in order to try to ameliorate the cognitive deficits that accompany these disorders (Toyohara and Hashimoto, 2010). Accordingly, antagonists of these nAChRs cause cognitive impairments in rodents, and hence certain drugs are used to mimic cognitive deficits seen in both Alzheimer's disease and schizophrenia.

Mecamylamine is such a non-selective nAChR antagonist shown to induce learning and memory deficits (at high enough doses) in rodents (Levin, 1992). In order to more specifically investigate the role of the different nAChR subtypes, selective nAChR antagonists are used. Methyllycaconitine (MLA) is a selective α7 nAChR competitive antagonist, and dihydro-beta-erythroidine (DHβE) is a selective α4β2 nAChR competitive antagonist. Both of these drugs have been shown to induce memory deficits in rodents (e.g. Addy et al., 2003), taken a high

enough dose is administered (Hahn et al., 2011). Besides from inducing cognitive deficits on their own, these drugs are also used to counteract the pro-cognitive effect of agonists at their corresponding nAChR subtype. This approach is used in order to confirm that the pro-cognitive effects of a selective nAChR agonist are indeed mediated via a specific nAChR (Hahn et al., 2011; Prickaerts et al., 2012).

Glutamatergic antagonists

Another important neurotransmitter directly involved in cognitive processes is glutamate. Glutamate is an abundantly present excitatory neurotransmitter, which acts through the ionotropic NMDA receptor (besides the AMPA receptor). NMDA receptors have been implicated in cognitive processes, in particular memory formation (van der Staay et al., 2011).

Following this rationale, NMDA antagonists have been used to function as cognition-deficit models in rodents and of these the most widely used cognition impairers are non-competitive NMDA receptor-channel blockers. The most frequently used NMDA receptor-channel blockers in rodent models are MK-801 (dizocilpine), phencyclidine (PCP) and ketamine. These receptor-channel blockers bind to specific sites within the NMDA receptor channel pore and subsequently block the channel, thereby inducing cognitive impairments.

MK-801 has been assessed in a broad range of rodent test paradigms and is considered a valid model to induce acute cognitive dysfunction provided the right dose is used (without inducing non-cognitive side-effects) (van der Staay et al., 2011). PCP in rodents is mainly used in a (sub)chronic manner to mimic the impairments seen in schizophrenic patients. In contrast to MK-801, PCP was also tested in humans, hence more direct comparisons between rodent- and human-behavior can be made (Ellison, 1995). PCP is believed to bind to a site within the NMDA receptor-channel pore (‘the PCP-binding site’) that is only accessible when the channel is open. Therefore, the antagonism is ‘use-dependent’. PCP thus acts at the same site as other ‘open channel’ blockers such as MK-801 or ketamine (Morris et al., 2005). Besides acting on the NMDA receptor channel, PCP also binds to the dopamine uptake site. MK-801 is considerable more potent than PCP in producing a non-competitive blockade at the NMDA receptor. However, MK-801 lacks the direct action on dopamine uptake, which accounts for the argument that PCP might be a more suitable deficit model for schizophrenia specifically, since PCP intoxication is associated with more psychotic features. Ketamine also acts as a type 2 dopamine partial agonist but is a weaker blocker of the NMDA ion channel. Therefore for mimicking

psychosis, PCP might represent a more (and ketamine a less) complete model. While MK-801 is much less complex in its pharmacological profile it has proved to be very valuable in animal studies because of its high selectivity for the NMDA receptor (Ellison, 1995). After scopolamine, MK-801 is probably the most widely used drug for the induction of cognitive impairments in rodents (van der Staay et al., 2011).

Serotonergic intervention

The serotonergic system has been implicated in cognitive processes as well. This system may have only minor effects on cognitive function on its own, but is assumed to interact with the cholinergic system. This serotonergic-cholinergic interaction probably plays an important role in the mediation of behavioral, including cognitive, performance (Steckler and Sahgal, 1995).

A model used to decrease serotonin (5-HT) entails the lowering of 5-HT levels. Decreasing 5-HT levels can be accomplished by manipulating the availability of the essential amino acid tryptophan via the food. Tryptophan has multiple functions, one of which is that it functions as a biochemical precursor for 5-HT. Acute tryptophan depletion is used as a pharmacological deficit model to lower central 5-HT levels. The acute tryptophan depletion method is widely used both preclinically and clinically as a model to investigate the implication of the 5-HT system in affective disorders (Booij et al., 2003; van Donkelaar et al., 2008). This serotonergic-deficit model has been frequently used to study putative cognition enhancers in rats (e.g. Rutten et al., 2007; van Donkelaar et al., 2008).

Aging and transgenic models

Over the past few decades, ample transgenic rodent models modeling specific diseases and exhibiting cognitive deficits have been generated. It should, however, be noted that most of the diseases discussed below are not of simple genetic origin. Indeed, the exact etiology of most remains to be elucidated. This means that the specific mutations used to create a model may only have a small hand in the actual pathology. Single mutations might not even result in any detectable pathology and multiple mutations, or specific environmental interactions may be required to instigate disease pathology (Chouliaras et al., 2010). Described in this section is a selection of transgenic rodent models of some of the most common neurodegenerative diseases involving cognitive impairments, which are most widely used or have provided critical insights.

Normal aging

Of most cognitive disorders aging is the top risk factor, while at the same time aging itself is also associated with cognitive decline (Gu et al., 2010). Although aging is a natural process, it can result in quite severe functional limitations at the end of the life-span, resulting inevitably in death. Rats and mice are useful laboratory species for studying the aging process, as they have relatively short lifespans (up 4 years for mice and up to 5 years for rats), are small and thus easy to keep, and reproduce fast (Gorbunova et al., 2008). For instance, non-transgenic mice can be used to study epigenetic, physiological, morphological and behavioral changes as they occur during the aging process (e.g. Rutten et al., 2010; Chouliaras et al., 2011). Importantly, interventions that may have a positive effect on age-related decline, such as caloric restriction, can be tested in these animals in a relatively short amount of time (Rutten et al., 2010). An even faster rodent model of aging is the senescence accelerated mouse (SAM). This model consists of a collection of series, created through the selective breeding of AKR/J mice, which already showed signs of accelerated aging, including multiple senescence-prone (P series) and senescence-resistant (R series) series (Takeda et al., 1981; Takeda et al., 1991). Of particular interest are the SAMP8 mice, which show ample age-related changes early in life, leading to a median survival time of only around 10 months. SAMP8 mice naturally present with neuropathological and neurochemical changes, including Aβ deposition, hyperphosphorylation of tau, hampered dendritic spine development, as well as NMDA, acetylcholine and noradrenalin associated abnormalities (Tomobe and Nomura, 2009). This makes the SAMP8 model attractive for the study of, for example, age-related Parkinson's and Alzheimer's disease (AD). At a young age SAMP8 mice already develop learning and memory impairments. Such deficits present themselves starting at 2 months of age, as assessed with such tests as the water maze, T-maze, passive avoidance and one-way active avoidance paradigms (Miyamoto et al., 1986; Tomobe and Nomura, 2009).

The greatest advantage of mice and rats may, however, also be one of their greatest limitations as models of human aging; the large gap between the lifespans of humans, and that of mice and rats, may be indicative of the latter being unable to fully elucidate the mechanisms influencing human aging (Gorbunova et al., 2008). For this reason some investigators have chosen to use animal models that live longer, among which are also other rodent models. Some of these model organisms, including the naked mole-rat, porcupines and beavers, reach lifespans of over 20 years. Comparing species of the same order of Rodentia with such diverging lifespans may offer insights into the general mechanisms that increase a species age.

Alzheimer's disease

APP

Despite its relative rarity, familial AD (fAD) has garnered the most attention due to its large genetic component. It is thus not surprising that the first transgenic mouse model for AD, the PDAPP model made in 1995, is based on a mutation in the fAD associated amyloid precursor protein (APP) gene (Nilsson et al., 2004; Giuliani et al., 2009). PDAPP mice express human APP cDNA with the Indiana mutation (V717F). In this model plaque pathology arises between 6-9 months, paired with synapse, but no severe cell-loss, or neurofibrillary tangle (NFT) deposition. Aged mice of this model display an impaired learning ability in the Morris water maze, the radial arm water maze, the cue task and serial spatial reversal task (Chen et al., 2007).

Although some neuropathology occurs in this first model, it is the second transgenic model, Tg2576, implementing a double APP mutation (K670N and M671L), that successfully models an age-dependent build-up of amyloid plaques and related cognitive decline, as associated with AD (King and Arendash, 2002). The mutant APP expressed by Tg2576 mice is also referred to as APPSWE, and is under control of the hamster prion promoter. Cognitive decline in these widely used mice occurs progressively from 6-9 months of age. By the age of 12 months this model shows an impaired performance on spatial and working memory tasks, including the Y-maze spontaneous alternation and visible platform recognition tasks, as well as amygdala-dependent fear conditioning tasks.

A more aggressive AD model, the TgCRND8 transgenic mouse model, combines the Swedish and Indiana mutations, expressing the human βAPP695 transgene under control of the Syrian hamster prion promoter, on a C3H/B6 background (Francis et al., 2012). This combination results in rapid extracellular plaque formation in the hippocampus and frontal cortex, similar to human AD, paired with defunct spatial learning in the Morris water maze task at 3 months of age, and impaired nonspatial episodic memory, as determined with the object recognition task at already 8 weeks of age.

PS1, PS2 and PS1 x APP

Apart from mutations in the APP gene, mutations in presenilin (PS) genes have also been used to generate transgenic mouse models. For instance, the PS1M146L, PS1M146V and PS2N141I models were used to demonstrate *in vivo* that mutant PS1 and PS2 are able to selectively enhance Aβ42 levels (McGowan et al., 2006). This increased Aβ42 presence is, however, without significant plaque pathology and cognitive deficits. It seems that the interaction between

the presenilin and APP genes is of vital importance in the pathophysiology of AD and therefore presenilin mutations are usually combined with a mutated APP transgene. The bigenic PSAPP model, a crossing between APP and PS1 transgenic models (e.g. Tg2576 x PS1M146L, PS1-A246 + APPSWE, APP_{swe}/PS1dE9), shows a grave acceleration in pathology, as compared to mutant APP-only models (Arendash et al., 2001). This includes an earlier onset of cognitive impairments, as measured with the Morris water maze and radial arm water maze tests for working memory.

One of the most early-onset and aggressive amyloid models is the 5XFAD transgenic mouse model, sporting five fAD associated mutations (Ohno et al., 2007). 5XFAD mice carry two transgenes under the mouse Thy-1 promoter; APPswe/Ind/fl and PS1M146L/L286V (on a B6/SJL background), resulting in a grossly exaggerated Aβ42 production. Consequently, amyloid deposits already start to form at the young age of 2 months in the hippocampus. By the age of 6 months, massive amyloid pathology can be observed throughout the hippocampus and cortex of these mice, paired with impaired spatial working memory, as tested with the spontaneous alternation Y-maze. At this age 5XFAD mice also show impaired hippocampal-dependent contextual fear memory (Kimura and Ohno, 2009).

MAPT

APP, PS1 and PS2 transgenic models are able to capture some of the Aβ-associated pathology seen in AD. Most of these models, however, fail to recapitulate the widespread neurodegeneration and tangle pathology, which is critical for a suitable phenocopy of AD. A model that achieves just that is the TauP301S transgenic mouse model, based on the shortest isoform of 4R microtubule associated protein tau (MAPT) with the P301S mutation, controlled by the mouse Thy-1 promoter on a C57BL/J background (Takeuchi et al., 2011). Around 5-6 months of age widespread NFT pathology can be observed in the brain and spinal cord, as well as neuronal loss in the latter area, paired with severe paraparesis in mice of this model. Cognitive deficits at 5 to 6 months of age include decreased spontaneous alternation in the Y-maze test, impaired sociability and object recognition memory in Crawley's social interaction test, hampered spatial memory in the Morris water maze test, and slightly impaired contextual memory in the contextual and cued fear conditioning tests.

The peculiar TauV337M model, which expresses 4R MAPT with the V337M mutation controlled by the platelet-derived growth factor promoter (also exists with the mouse Thy-1 promoter) on a B6SJL background, is characterized by a

low level synthesis of 4R MAPT that is only 1/10 of endogenous mouse MAPT production (Lambourne et al., 2005). The observation of neurofibrillary pathology in this model indicates that it may not be the absolute MAPT levels, but the nature of MAPT that instigates tangle pathology. At the age of 12 months, TauV337M mice seem to have defunct olfactory memory, as tested with the social transmission of food preferences task, and deficits in impulse control, as determined with the 5-choice serial reaction time task, at 24 months of age and at 12 months of age when the inter-trial intervals were increased. Note that in contrast to most other tau-based models, this model does not exhibit motor abnormalities until at least 24 months of age.

To investigate the reversibility of tangle pathology, the rTg4510 model was created (Santacruz et al., 2005). These transgenic mice express MAPT with the P301L mutation under control of the TET-off system, making the transgene inducible. When the mutant MAPT is expressed, these mice show progressive NFT development and cell loss from one month of age, including severe hippocampal CA1 neuron death at the age of 5 months. From an age of 2.5 months this model starts to display impaired spatial reference memory, as examined with the Morris water maze. Interestingly, turning off production of the mutant MAPT after 4 months of age leads to a recovery of cognitive performance, but a worsening of the tangle pathology, indicating that at this age tau pathology becomes independent of transgenic MAPT expression.

When considering the MAPT based models discussed above as models for AD it is important to realize that NFT pathology in AD arises in the absence of mutations in the MAPT gene; indeed, most of the mutations these models are based on are from other tauopathies such as frontotemporal dementia (FTD) (McGowan et al., 2006). Furthermore, most of the other transgenic models do not take into account endogenous gene expression of the model organism. For instance, all of the above transgenic mouse models that express a mutant form of MAPT also express mouse MAPT in addition. The htau transgenic mouse model was created with these points in mind; expressing non-mutant human genomic MAPT in a mouse MAPT knock-out background (maintained on a Swiss Webster/129/SvJae/C57BL/6 background) (Andorfer et al., 2003). This model presents with AD-like tau pathology, starting with pre-tangle-like hyperphosphorylated MAPT accumulation after 3 months, spreading at an age of 9 months through hippocampal and neocortical regions. At the age of 12 months these htau mice start to display cognitive impairments in the object recognition task and the Morris water maze, paired with disrupted long-term potentiation in the hippocampal CA1 region (Polydoro et al., 2009).

PS1 x APP x MAPT

One of the most used transgenic models for AD is the triple transgenic mouse model, which combines mutated PS1, APP and MAPT genes into one model. This 3xTgAD model expresses mutant APPSWE and MAPTP301L, under control of the mouse Thy-1 promoter, on a PSEN1M146V knock-in background (PSEN1-KI) (Oddo et al., 2003). Plaques develop from an age of 6 months in 3xTgAD mice, and tangle pathology arises by the age of 12 months. Although not completely mimicking AD, this is one of the best models available; developing progressive synaptic dysfunction, amyloid plaques and neurofibrillary tangles in a temporal and spatial pattern that is similar to human AD. Around 4 months of age 3xTgAD mice start to present with impaired spatial memory and long-term retention, as tested with the Morris water maze task, and at 6 months also their short- and long-term retention for contextual fear becomes significantly reduced (Billings et al., 2005). Aged 3xTgAD mice show deficits in object discrimination memory in the object discrimination task, together with derailed long-term potentiation and paired-pulse facilitation.

APOE4

When looking at the genes used in the triple transgenic AD model, it can be argued that it is primarily a model of fAD and not of the far more common sporadic AD (sAD). Models employing the highest genetic risk factor for sAD, allele APOE4, have been constructed; expressing human APOE4 under control of the neuron-specific enolase (NSE) promoter in transgenic mice devoid of endogenous mouse APOE (Raber et al., 1998). This NSE-APOE4 model exhibits a less severe phenotype than most other transgenic models of AD, failing to recapitulate most of the pathological hallmarks associated with the disease. Nevertheless, the NSE-APOE4 model displays impaired excitatory synaptic transmission, a decline in dendritic density and complexity, and cognitive impairments in a water maze task at an age of 6 months.

Parkinson's disease

α-Syn

α-Synuclein transgenic mice overexpress human wild type or mutant alpha-synuclein usually under the regulatory control of the human PDGF-ß promoter. *α*-Synuclein is expressed in high levels, resulting in an age-dependent increase of brain inclusions consisting of *α*-Synuclein (*α*-Syn), ubiquitin and other proteins. Severity of the brain pathology correlates with increasing age. By six months of age these transgenic mice exhibit deficits in cognition shown by an increased time to find the platform in the water maze task (Masliah et al., 2011). Mice

overexpressing wild type α-Syn under regulation of the human PDGF-β promoter also display a progressive increase in α-Syn aggregation in multiple brain regions, a loss of dopaminergic terminals in the striatum and mild changes in motor activity as shown by a decreased latency to fall on a rotarod. Another variation of these mice uses the human Thy1 as a promoter for overexpressing α-Syn. Cognitive changes (Y-maze, novel object recognition, and operant reversal learning) are also evident in the Thy1-α-Syn mice beginning around four to six months of age (Fleming et al., 2008; Magen and Chesselet, 2010).

Nuber et al. in 2008 created a conditional mouse model for the overexpression of WT α-Syn under the calcium/ calmodulin-dependent protein kinase IIα (CaM) promoter, using a tetracycline-regulated TET-off system (tTA). These mice displayed a progressive motor decline after 7 months (rotarod), modest impairment in reference memory after 12 months (water maze), α-Syn accumulation in the substantia nigra, hippocampus and olfactory bulb (Nuber et al., 2008).

DJ1(PARK7)KO
DJ1KO mice have a deficiency in expressing the Park7 protein, due to a knockout of the respective gene, namely DJ1. DJ1−/− mice between 13 and 14 months of age show cognitive deficits, as characterized by reduced performance in an object recognition task (Pham et al., 2010).

Parkin(PARK2)KO
This PD mouse model is produced by a knockout in the PARK2 gene, responsible for the expression of a protein called parkin. Parkin−/− mice display increased anxiety, as shown in open field and light/dark preference tests, and cognitive impairment exhibited as spatial memory deficits in the Morris water maze (Zhu et al., 2007). Mice that lack exon 3 in the parkin gene do not demonstrate loss of dopaminergic neurons, nevertheless they show signs of altered synaptic transmission in the nigrostriatal circuit (Goldberg et al., 2003).

Huntington's disease
Various transgenic rodent models of HD have been found to exhibit affective and cognitive abnormalities reflecting clinical data in HD patients. For example, R6/1 and R6/2 transgenic lines of HD mice have behavioral deficits that include impaired hippocampal-dependent spatial cognition (Pang et al., 2006; Nithianantharajah et al., 2008). However, depressive-like behavior also

manifests in R6/1 HD mice prior to cognitive and motor symptoms (Pang et al., 2009).

R6/2

Of the transgenic chimeric models that express truncated forms of the human mutant HD allele, the R6/2 line is the most widely used. This line expresses an exon 1 fragment of htt with a range of 148–153 repeats, expressed from an unknown location in the mouse genome. R6/2 mice exhibit learning and memory tasks abnormalities as early as 3.5 weeks of age (water Morris maze), which follow them throughout their lifespan, as evaluated by various cognitive tests (T-maze, two choice swim tank, visual discriminate learning) (Carter et al., 1999; Lione et al., 1999; Stack et al., 2005). Moreover, they show behavioral deficits by 5 weeks, neuroanatomic abnormalities including progressive reduction in brain and striatal volume, substantially reduced striatal neuron number by 12 weeks, and death by 12–15 weeks (Hickey et al., 2005; Morton et al., 2005; Stack et al., 2005). As the R6/2 model exhibits severe, early onset and diffuse pathology, it is potentially a good model of juvenile-onset HD, displaying an aggressive phenotype and provides clear experimental endpoints.

YAC128

The YAC128 is a widely used yeast-artificial-chromosome full length human mutant HD transgenic model generated and characterized by the Hayden laboratory (Slow et al., 2003; van Raamsdonk et al., 2005). van Raamsdonk et al. in 2005 evaluated YAC128 mice with a variety of more cognitively oriented tests, demonstrating progressive cognitive deficits at 8 weeks (accelerated rotarod) and 32 weeks (water Morris maze, open field habituation, and T-maze). Unlike the R6/2 mice, where there is probably a diffuse loss of brain volume, some regions of the YAC128 brain, such as the cerebellum and hippocampus, exhibit normal volume (van Raamsdonk et al., 2005). YAC128 mice also exhibit motor abnormalities as early as 3 months with increased open field activity, followed by rotarod performance abnormalities at 6 months.

tgHD rats

This transgenic rat model of HD, with a mutated huntingtin gene containing 51 CAG repeats, expresses adult-onset neurological phenotypes, cognitive impairments, progressive motor dysfunction and neuronal nuclear inclusions in the brain (von Horsten et al., 2003). The transgenic rat model exhibits a late onset neurological phenotype, cognitive decline in spatial learning at 10 months (radial arm maze) and significantly impaired object recognition performance at 16 months (Zeef et al., 2012), develops gradually progressive motor

abnormalities and dies between 15 and 24 months. However, according to a recent report by Fielding et al. in 2012, the tgHD rat model does not show consistent, reliable and progressive impairment in a range of cognitive tests (Fielding et al., 2012). The consistent failure to reveal impairments at any age on a range of tests of cognition and learning suggest that the tgHD rat is not a reliable model of the cognitive and behavioral impairments of human HD.

Frontotemporal dementia

TDP43

Transgenic murine models used in FTLD-TDP research overexpress either wild type or mutant human TDP43KI via a human TDP promoter.TDP43 is a multifunctional, nuclear protein that binds both DNA and RNA, as well as a member of the heterogeneous nuclear ribonuclear protein (hnRNP) family and regulates several aspects of RNA processing, including alternative splicing, miRNA production, and mRNA trafficking and stabilization. Missense changes in the glycine-rich domain of TDP-43 lead to a shift in its localization from the nucleus to the cytoplasm, resulting in FTLD-TDP pathology (Swarup et al., 2011). This mouse model exhibits cognitive deficits at the age of 7 or 9 months, depending on the use of a mutated or a wild type TDP43KI, respectively, as shown by passive avoidance test and Barns maze. Cognitive impairments for this murine model reach a peak at the age of 11-13 months (Tsai et al., 2010; Swarup et al., 2011).

Down syndrome

TgDyrk1A

Apart from the trisomic mice used in Down syndrome research, transgenic models have also been constructed carrying human genes mapped in the repeated fragment of chromosome 21. One such model, namely TgDyrk1a, overexpresses DYRK1A, a gene encoding a serine-threonine kinase, which is probably involved in neuroblast proliferation (Altafaj et al., 2001). In the Morris water maze, TgDyrk1A mice show significant deficits in spatial learning and cognitive flexibility, due to hippocampal and prefrontal cortical dysfunction, a defect that was related specifically to reference memory. TgDyrk1A mice also exhibit delayed craniocaudal maturation, altered motor skill acquisition and hyperactivity (Ahn et al., 2006).

Translation to clinics: limitations and difficulties

To date, pharmacological, transgenic and naturally aging rodent models have provided new insights into behavioral function. Although these models have given invaluable information, it is important to remember that they only provide approximations of the molecular and cellular mechanisms and cognitive impairments as seen in humans. In addition, while motor phenotypes can be readily assessed in rodent models, it is more challenging to characterize cognitive phenotypes. The frontal cortex of rodents is anatomically different from that of humans (Uylings et al., 2003), and it is therefore difficult to model executive dysfunction, not to mention the existence of significant limitations in modeling complex behaviors in rats and mice, since they already differ in their own cognitive and social functions. Due to these obstacles face validity gets compromised (see Table 1). It has thus been suggested that the research community should take an "agnostic" approach as new models emerge and characterize their behavior as fully as possible. As it is now, face validity of the behavioral tests used, is in general the same for pharmacological, aging and transgenic rodent models (see Table 2 and 3). This results from the fact that, independent of the manipulation used (pharmacological, age or genetic), the same symptoms are being screened for. In addition, it is important to recognize that not all animal models currently in use or under development will be appropriate for mechanistic research, whereas other certain models exhibit phenotypes that are practical for therapy development. When compared to pharmacological models, transgenic models comprise more construct validity, and hence are more suited for mechanistic and fundamental research. On the other hand, pharmacological models comprise more predictive validity, and therefore contribute to more reliable testing of new (pharmacological) therapies (see Table 2 and 3). Subjecting rodents to a comprehensive battery of tests provides a better framework for understanding not only the overall behavioral phenotype of the animal, but also for more fully recognizing the limitations of the specific model. Nevertheless, it should be emphasized that the goal of animal research is to mimic as much as possible the human disease pathophysiology, and thus improving construct and face validity will provide greater insights into basic genetic and molecular mechanisms involved in the expression of behavior (D'Mello and Steckler, 1996). At the same time, once these mechanisms are better understood, predictive validity will improve and better efficacious therapeutic strategies can be explored and developed for treating cognitive deficits in human patients.

Table 1: Descriptions of construct, face, and predictive validity

Validities	Description
Validity	The extent to which a test measures what it purports to measure. It is vital for a test to be valid in order for the results to be accurately applied and interpreted.
Construct validity	This is in generally considered the most fundamental and all-inclusive validity concept, insofar as it specifies what the test measures. Construct validity holds that the model has a correct theoretical background compared to the human pathology. Therefore, it addresses the qualities contributing to the relation between *X* and *Y*. Overall, construct validity deals with the question: Does the measure or observation in a test or model show behavior that corresponds to how the theory says a measure or observation of that construct should behave?
Face validity	The extent to which a test seems on its surface to be measuring what it purports to measure. Face validity refers not to what the test measures but only to how it looks. The concept of face validity is that the animal shows the same kind of behavior and has the same symptoms as humans have. In short; do the measures in a test or model *appear* to be relevant?
Predictive validity	The extent to which a test or model can predict future outcomes. Predictive validity implies that the manipulations and treatments that are beneficial in the appropriate animal model should also have the same effect in humans/patients, and vice versa.

Table 2: Validity of pharmacological models

Validities / Pharmacological models	Construct validity	Face validity	Predictive validity
Inhibition of energy/glucose metabolism	+++	++	+
Cholinergic toxins	+++	++	+
Cholinergic antagonists	++	++	++
Glutamatergic antagonists	++	++	++
Serotonergic intervention	+	++	+

++++: meets validity perfectly, +++: meets validity good, ++: meets validity somewhat, +: meets validity poorly

Table 3: Validity of aging and transgenic models in general

Validities / Aging and transgenic models	Construct validity	Face validity	Predictive validity
Normal Aging	++++	++	++
Alzheimer's Disease models	+++	++	+
Parkinson's Disease models	+++	++	+
Huntinton's Disease models	+++	++	+
Frontotemporal Dementia TDP43 model	+++	++	+
Down SyndromeTgDyrk model	+++	++	+

++++: meets validity perfectly, +++: meets validity good, ++: meets validity somewhat, +: meets validity poorly

References

Addy, N. A., Nakijama, A., and Levin, E. D. (2003). Nicotinic mechanisms of memory: effects of acute local DHβE and MLA infusions in the basolateral amygdala. *Cognitive Brain Research, 16*(1), 51-57.

Ahn, K.-J., Jeong, H. K., Choi, H.-S., Ryoo, S.-R., Kim, Y. J., Goo, J.-S., et al. (2006). DYRK1A BAC transgenic mice show altered synaptic plasticity with learning and memory defects. *Neurobiology of Disease, 22*(3), 463-472.

Altafaj, X., Dierssen, M., Baamonde, C., Marti, E., Visa, J., Guimera, J., et al. (2001). Neurodevelopmental delay, motor abnormalities and cognitive deficits in transgenic mice overexpressing Dyrk1A (minibrain), a murine model of Down's syndrome. *Human Molecular Genetics, 10*(18), 1915-1923.

Andorfer, C., Kress, Y., Espinoza, M., de Silva, R., Tucker, K. L., Barde, Y. A., et al. (2003). Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. *Journal of Neurochemistry, 86*(3), 582-590.

Arendash, G. W., King, D. L., Gordon, M. N., Morgan, D., Hatcher, J. M., Hope, C. E., et al. (2001). Progressive, age-related behavioral impairments in transgenic mice carrying both mutant amyloid precursor protein and presenilin-1 transgenes. *Brain Research, 891*(1-2), 42-53.

Arias, R. L., Tasse, J. R. P., and Bowlby, M. R. (1999). Neuroprotective interaction effects of NMDA and AMPA receptor antagonists in an in vitro model of cerebral ischemia. *Brain Research, 816*(2), 299-308.

Bartus, R. T., Dean, R. L., Beer, B., and Lippa, A. S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science, 217*, 408-417.

Bederson, J. B., Pitts, L. H., Tsuji, M., Nishimura, M., Davis, R., and Bartkowski, H. (1986). Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke, 17*(3), 472-476.

Billings, L. M., Oddo, S., Green, K. N., McGaugh, J. L., and LaFerla, F. M. (2005). Intraneuronal Abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron, 45*(5), 675-688.

Blokland, A. (1995). Acetylcholine: a neurotransmitter for learning and memory? *Brain Research Reviews, 21*(3), 285-300.

Blokland, A., and Jolles, J. (1993). Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. *Pharmacology Biochemistry and Behavior, 44*(2), 491-494.

Booij, L., van der Does, A., and Riedel, W. (2003). Monoamine depletion in psychiatric and healthy populations: review. *Molecular Psychiatry, 8*(12), 951-973.

Book, A. A., Wiley, R. G., and Schweitzer, J. B. (1992). Specificity of 192 IgG-saporin for NGF receptor-positive cholinergic basal forebrain neurons in the rat. *Brain Research, 590*(1), 350-355.

Carter, R. J., Lione, L. A., Humby, T., Mangiarini, L., Mahal, A., Bates, G. P., et al. (1999). Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. *Journal of Neuroscience, 19*(8), 3248-3257.

Caulfield, M. P. (1993). Muscarinic receptors—characterization, coupling and function. *Pharmacology & Therapeutics, 58*(3), 319-379.

Chen, G., Chen, K. S., Kobayashi, D., Barbour, R., Motter, R., Games, D., et al. (2007). Active β-amyloid immunization restores spatial learning in PDAPP mice displaying very low levels of β-amyloid. *Journal of Neuroscience, 27*(10), 2654-2662.

Cho, S., Wood, A., and Bowlby, M. R. (2007). Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics. *Current Neuropharmacology, 5*(1), 19-33.

Chouliaras, L., Sierksma, A. S., Kenis, G., Prickaerts, J., Lemmens, M. A., Brasnjevic, I., et al. (2010). Gene-environment interaction research and transgenic mouse models of Alzheimer's disease. *International Journal of Alzheimer's Disease, 2010*, 1-27.

Chouliaras, L., van den Hove, D., Kenis, G., Dela Cruz, J., Lemmens, M., van Os, J., et al. (2011). Caloric restriction attenuates age-related changes of DNA methyltransferase 3a in mouse hippocampus. *Brain, Behavior, and Immunity, 25*(4), 616-623.

Chouliaras, L., van den Hove, D. L., Kenis, G., Keitel, S., Hof, P. R., van Os, J., et al. (2012). Prevention of age-related changes in hippocampal levels of 5-methylcytidine by caloric restriction. *Neurobiology of Aging, 33*(8), 1672-1681.

D'Mello, G. D., and Steckler, T. (1996). Animal models in cognitive behavioural pharmacology: an overview. *Cognitive Brain Research, 3*(3-4), 345-352.

Danysz, W., and Parsons, C. G. (2003). The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *International Journal of Geriatric Psychiatry, 18*(S1), S23-S32.

Ding, A., Nitsch, R., and Hoyer, S. (1992). Changes in brain monoaminergic neurotransmitter concentrations in rat after intracerebroventricular injection of streptozotocin. *Journal of Cerebral Blood Flow and Metabolism, 12*, 103-109.

Ellison, G. (1995). The N-methyl-d-aspartate antagonists phencyclidine, ketamine and dizocilpine as both behavioral and anatomical models of the dementias. *Brain Research Reviews, 20*(2), 250-267.

Faigle, R., and Song, H. (2013). Signaling mechanisms regulating adult neural stem cells and neurogenesis. *Biochimica et Biophysica Acta, 1830*(2), 2435-2448.

Fielding, S. A., Brooks, S. P., Klein, A., Bayram-Weston, Z., Jones, L., and Dunnett, S. B. (2012). Profiles of motor and cognitive impairment in the transgenic rat model of Huntington's disease. *Brain Research Bulletin, 88*(2-3), 223-236.

Fleming, S. M., Garcia, E., Masliah, E., Chesselet, M. F., and Jentsch, J. D. (2008). Impaired reversal learning in transgenic mice overexpressing human wildtype alpha synuclein. *Neuroscience Abstracts, 341.1*.

Francis, B. M., Kim, J., Barakat, M. E., Fraenkl, S., Yucel, Y. H., Peng, S., et al. (2012). Object recognition memory and BDNF expression are reduced in young TgCRND8 mice. *Neurobiology of Aging, 33*(3), 555-563.

Froestl, W., Muhs, A., and Pfeifer, A. (2012). Cognitive Enhancers (Nootropics). Part 1: Drugs Interacting with Receptors. *Journal of Alzheimer's Disease, 32*, 793-887.

Giuliani, F., Vernay, A., Leuba, G., and Schenk, F. (2009). Decreased behavioral impairments in an Alzheimer mice model by interfering with TNF-alpha metabolism. *Brain Research Bulletin, 80*(4-5), 302-308.

Gold, P. E. (1995). Role of glucose in regulating the brain and cognition. *The American Journal of Clinical Nutrition, 61*(4), 987S-995S.

Goldberg, M. S., Fleming, S. M., Palacino, J. J., Cepeda, C., Lam, H. A., Bhatnagar, A., et al. (2003). Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *Journal of Biological Chemistry, 278*(44), 43628-43635.

Gorbunova, V., Bozzella, M. J., and Seluanov, A. (2008). Rodents for comparative aging studies: from mice to beavers. *Age, 30*(2-3), 111-119.

Gu, Y., Huang, C. S., Inoue, T., Yamashita, T., Ishida, T., Kang, K. M., et al. (2010). Drinking hydrogen water ameliorated cognitive impairment in senescence-accelerated mice. *Journal of Clinical Biochemistry and Nutrition, 46*(3), 269-276.

Hahn, B., Shoaib, M., and Stolerman, I. P. (2011). Selective nicotinic receptor antagonists: effects on attention and nicotine-induced attentional enhancement. *Psychopharmacology, 217*(1), 75-82.

Hellweg, R., Nitsch, R., Hock, C., Jaksch, M., and Hoyer, S. (1992). Nerve growth factor and choline acetyltransferase activity levels in the rat brain following experimental impairment of cerebral glucose and energy metabolism. *Journal of Neuroscience Research, 31*(3), 479-486.

Hickey, M., Gallant, K., Gross, G., Levine, M., and Chesselet, M.-F. (2005). Early behavioral deficits in R6/2 mice suitable for use in preclinical drug testing. *Neurobiology of Disease, 20*(1), 1-11.

Kimura, R., and Ohno, M. (2009). Impairments in remote memory stabilization precede hippocampal synaptic and cognitive failures in 5XFAD Alzheimer mouse model. *Neurobiology of Disease, 33*(2), 229-235.

King, D. L., and Arendash, G. W. (2002). Behavioral characterization of the Tg2576 transgenic model of Alzheimer's disease through 19 months. *Physiology & Behavior, 75*(5), 627-642.

Klinkenberg, I., and Blokland, A. (2010). The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies. *Neuroscience and Biobehavioral Reviews, 34*(8), 1307-1350.

Klinkenberg, I., and Blokland, A. (2011). A comparison of scopolamine and biperiden as a rodent model for cholinergic cognitive impairment. *Psychopharmacology, 215*(3), 549-566.

Korol, D. L., and Gold, P. E. (1998). Glucose, memory, and aging. *The American Journal of Clinical Nutrition, 67*(4), 764S-771S.

Lambourne, S. L., Sellers, L. A., Bush, T. G., Choudhury, S. K., Emson, P. C., Suh, Y.-H., et al. (2005). Increased tau phosphorylation on mitogen-activated protein kinase consensus sites and cognitive decline in transgenic models for Alzheimer's disease and FTDP-17: evidence for distinct molecular processes underlying tau abnormalities. *Molecular and Cellular Biology, 25*(1), 278-293.

Lehmann, O., Grottick, A., Cassel, J. C., and Higgins, G. (2003). A double dissociation between serial reaction time and radial maze performance in rats subjected to 192 IgG-saporin lesions of the nucleus basalis and/or the septal region. *European Journal of Neuroscience, 18*(3), 651-666.

Levin, E. D. (1992). Nicotinic systems and cognitive function. *Psychopharmacology, 108*(4), 417-431.

Lione, L. A., Carter, R. J., Hunt, M. J., Bates, G. P., Morton, A. J., and Dunnett, S. B. (1999). Selective discrimination learning impairments in mice expressing the human Huntington's disease mutation. *Journal of Neuroscience, 19*(23), 10428-10437.

Magen, I., and Chesselet, M. F. (2010). Genetic mouse models of Parkinson's disease The state of the art. *Progress in Brain Research, 184*, 53-87.

Masliah, E., Rockenstein, E., Mante, M., Crews, L., Spencer, B., Adame, A., et al. (2011). Passive immunization reduces behavioral and neuropathological deficits in an alpha-synuclein transgenic model of Lewy body disease. *PloS One, 6*(4), e19338.

Mayer, G., Nitsch, R., and Hoyer, S. (1990). Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats. *Brain Research, 532*(1), 95-100.

McGowan, E., Eriksen, J., and Hutton, M. (2006). A decade of modeling Alzheimer's disease in transgenic mice. *Trends in Genetics : TIG, 22*(5), 281-289.

Miyamoto, M., Kiyota, Y., Yamazaki, N., Nagaoka, A., Matsuo, T., Nagawa, Y., et al. (1986). Age-related changes in learning and memory in the senescence-accelerated mouse (SAM). *Physiology & Behavior, 38*(3), 399-406.

Morris, B. J., Cochran, S. M., and Pratt, J. A. (2005). PCP: from pharmacology to modelling schizophrenia. *Current Opinion in Pharmacology, 5*(1), 101-106.

Morton, A. J., Hunt, M. J., Hodges, A. K., Lewis, P. D., Redfern, A. J., Dunnett, S. B., et al. (2005). A combination drug therapy improves cognition and reverses gene expression changes in a mouse model of Huntington's disease. *The European Journal of Neuroscience, 21*(4), 855-870.

Nilsson, L. N., Arendash, G. W., Leighty, R. E., Costa, D. A., Low, M. A., Garcia, M. F., et al. (2004). Cognitive impairment in PDAPP mice depends on ApoE and ACT-catalyzed amyloid formation. *Neurobiology of Aging, 25*(9), 1153-1167.

Nithianantharajah, J., Barkus, C., Murphy, M., and Hannan, A. J. (2008). Gene–environment interactions modulating cognitive function and molecular correlates of synaptic plasticity in Huntington's disease transgenic mice. *Neurobiology of Disease, 29*(3), 490-504.

Nitsch, R., Mayer, G., and Hoyer, S. (1989). The intracerebroventriculary streptozotocin-treated rat: Impairment of cerebral glucose metabolism resembles the alterations of carbohydrate metabolism of the brain in Alzheimer's disease. *Journal of Neural Transmission: Parkinson's Disease and Dementia Section, 1*(1), 109-110.

Nuber, S., Petrasch-Parwez, E., Winner, B., Winkler, J., von Horsten, S., Schmidt, T., et al. (2008). Neurodegeneration and motor dysfunction in a conditional model of Parkinson's disease. *Journal of Neuroscience, 28*(10), 2471-2484.

Ockuly, J. C., Gielissen, J. M., Levenick, C. V., Zeal, C., Groble, K., Munsey, K., et al. (2012). Behavioral, cognitive, and safety profile of 2-deoxy-2-glucose (2DG) in adult rats. *Epilepsy Research, 101*, 246-252.

Oddo, S., Caccamo, A., Kitazawa, M., Tseng, B. P., and LaFerla, F. M. (2003). Amyloid deposition precedes tangle formation in a triple transgenic model of Alzheimer's disease. *Neurobiology of Aging, 24*(8), 1063-1070.

Ohno, M., Cole, S. L., Yasvoina, M., Zhao, J., Citron, M., Berry, R., et al. (2007). BACE1 gene deletion prevents neuron loss and memory deficits in 5XFAD APP/PS1 transgenic mice. *Neurobiology of Disease, 26*(1), 134-145.

Pang, T., Stam, N., Nithianantharajah, J., Howard, M., and Hannan, A. (2006). Differential effects of voluntary physical exercise on behavioral and brain-derived neurotrophic factor expression deficits in Huntington's disease transgenic mice. *Neuroscience, 141*(2), 569-584.

Pang, T. Y., Du, X., Zajac, M. S., Howard, M. L., and Hannan, A. J. (2009). Altered serotonin receptor expression is associated with depression-related behavior in the R6/1 transgenic mouse model of Huntington's disease. *Human Molecular Genetics, 18*(4), 753-766.

Peternel, A., Hughey, D., Wenk, G. L., and Olton, D. (1988). Basal forebrain and memory: Neurotoxic lesions impair serial reversals of a spatial discrimination. *Psychobiology, 16*, 54-58.

Pham, T., Giesert, F., Röthig, A., Floss, T., Kallnik, M., Weindl, K., et al. (2010). DJ-1-deficient mice show less TH-positive neurons in the ventral tegmental area and exhibit non-motoric behavioural impairments. *Genes, Brain and Behavior, 9*(3), 305-317.

Polydoro, M., Acker, C. M., Duff, K., Castillo, P. E., and Davies, P. (2009). Age-dependent impairment of cognitive and synaptic function in the htau mouse model of tau pathology. *Journal of Neuroscience, 29*(34), 10741-10749.

Prickaerts, J., Blokland, A., Honig, W., Meng, F., and Jolles, J. (1995). Spatial discrimination learning and choline acetyltransferase activity in streptozotocin-treated rats: effects of chronic treatment with acetyl-L-carnitine. *Brain Research, 674*(1), 142-146.

Prickaerts, J., van Goethem, N. P., Chesworth, R., Shapiro, G., Boess, F. G., Methfessel, C., et al. (2012). EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacology, 62*, 1099-1110.

Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R. E., et al. (1998). Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: increased susceptibility of females. *Proceedings of the National Academy of Sciences of the United States of America, 95*(18), 10914-10919.

Rutten, B. P., Brasnjevic, I., Steinbusch, H. W., and Schmitz, C. (2010). Caloric restriction and aging but not overexpression of SOD1 affect hippocampal volumes in mice. *Mechanisms of Ageing and Development, 131*(9), 574-579.

Rutten, K., Lieben, C., Smits, L., and Blokland, A. (2007). The PDE4 inhibitor rolipram reverses object memory impairment induced by acute tryptophan depletion in the rat. *Psychopharmacology, 192*(2), 275-282.

Santacruz, K., Lewis, J., Spires, T., Paulson, J., Kotilinek, L., Ingelsson, M., et al. (2005). Tau suppression in a neurodegenerative mouse model improves memory function. *Science, 309*(5733), 476-481.

Slow, E. J., van Raamsdonk, J., Rogers, D., Coleman, S. H., Graham, R. K., Deng, Y., et al. (2003). Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Human Molecular Genetics, 12*(13), 1555-1567.

Stack, E. C., Kubilus, J. K., Smith, K., Cormier, K., Del Signore, S. J., Guelin, E., et al. (2005). Chronology of behavioral symptoms and neuropathological sequela in R6/2 Huntington's disease transgenic mice. *The Journal of Comparative Neurology, 490*(4), 354-370.

Steckler, T., Andrews, J. S., Marten, P., and Turner, J. D. (1993). Effects of NBM lesions with two neurotoxins on spatial memory and autoshaping. *Pharmacology Biochemistry and Behavior, 44*(4), 877-889.

Steckler, T., and Sahgal, A. (1995). The role of serotonergic-cholinergic interactions in the mediation of cognitive behaviour. *Behavioural Brain Research, 67*(2), 165-199.

Swarup, V., Phaneuf, D., Bareil, C., Robertson, J., Rouleau, G. A., Kriz, J., et al. (2011). Pathological hallmarks of amyotrophic lateral sclerosis/frontotemporal lobar degeneration in transgenic mice produced with TDP-43 genomic fragments. *Brain, 134*(Pt 9), 2610-2626.

Takeda, T., Hosokawa, M., and Higuchi, K. (1991). Senescence-accelerated mouse (SAM): a novel murine model of accelerated senescence. *Journal of the American Geriatrics Society, 39*(9), 911-919.

Takeda, T., Hosokawa, M., Takeshita, S., Irino, M., Higuchi, K., Matsushita, T., et al. (1981). A new murine model of accelerated senescence. *Mechanisms of Ageing and Development, 17*(2), 183-194.

Takeuchi, H., Iba, M., Inoue, H., Higuchi, M., Takao, K., Tsukita, K., et al. (2011). P301S mutant human tau transgenic mice manifest early symptoms of human tauopathies with dementia and altered sensorimotor gating. *PloS One, 6*(6), e21050.

Tomobe, K., and Nomura, Y. (2009). Neurochemistry, neuropathology, and heredity in SAMP8: a mouse model of senescence. *Neurochemical Research, 34*(4), 660-669.

Toyohara, J., and Hashimoto, K. (2010). α7 Nicotinic receptor agonists: potential therapeutic drugs for treatment of cognitive impairments in schizophrenia and Alzheimer's disease. *The Open Medicinal Chemistry Journal, 4*, 37-56.

Tsai, K.-J., Yang, C.-H., Fang, Y.-H., Cho, K.-H., Chien, W.-L., Wang, W.-T., et al. (2010). Elevated expression of TDP-43 in the forebrain of mice is sufficient to cause neurological and pathological phenotypes mimicking FTLD-U. *The Journal of Experimental Medicine, 207*(8), 1661-1673.

Uylings, H., Groenewegen, H. J., and Kolb, B. (2003). Do rats have a prefrontal cortex? *Behavioural Brain Research, 146*(1), 3-17.

van der Staay, F. J., Rutten, K., Erb, C., and Blokland, A. (2011). Effects of the cognition impairer MK-801 on learning and memory in mice and rats. *Behavioural Brain Research, 220*(1), 215-229.

van Donkelaar, E. L., Rutten, K., Blokland, A., Akkerman, S., Steinbusch, H. W., and Prickaerts, J. (2008). Phosphodiesterase 2 and 5 inhibition attenuates the object memory deficit induced by acute tryptophan depletion. *European Journal of Pharmacology, 600*(1), 98-104.

van Raamsdonk, J. M., Pearson, J., Bailey, C. D., Rogers, D. A., Johnson, G. V., Hayden, M. R., et al. (2005). Cystamine treatment is neuroprotective in the YAC128 mouse model of Huntington disease. *Journal of Neurochemistry, 95*(1), 210-220.

van Raamsdonk, J. M., Pearson, J., Slow, E. J., Hossain, S. M., Leavitt, B. R., and Hayden, M. R. (2005). Cognitive dysfunction precedes neuropathology and motor abnormalities in the YAC128 mouse model of Huntington's disease. *Journal of Neuroscience, 25*(16), 4169-4180.

Vavra, J., DeBoer, C., Dietz, A., Hanka, L., & Sokolski, W. (1959). Streptozotocin, a new antibacterial antibiotic. *Antibiotics Annual, 7*, 230-235.

von Horsten, S., Schmitt, I., Nguyen, H. P., Holzmann, C., Schmidt, T., Walther, T., et al. (2003). Transgenic rat model of Huntington's disease. *Human Molecular Genetics, 12*(6), 617-624.

Walsh, T., Herzog, C., Gandhi, C., Stackman, R., and Wiley, R. (1996). Injection of IgG 192-saporin into the medial septum produces cholinergic hypofunction and dose-dependent working memory deficits. *Brain Research, 726*(1), 69-79.

Wenk, G. L., Stoehr, J. D., Quintana, G., Mobley, S., and Wiley, R. G. (1994). Behavioral, biochemical, histological, and electrophysiological effects of 192 IgG-saporin injections into the basal forebrain of rats. *Journal of Neuroscience, 14*(10), 5986-5995.

Wiley, R. G., Oeltmann, T. N., and Lappi, D. A. (1991). Immunolesioning: selective destruction of neurons using immunotoxin to rat NGF receptor. *Brain Research, 562*(1), 149-153.

Zeef, D. H., van Goethem, N. P., Vlamings, R., Schaper, F., Jahanshahi, A., Hescham, S., et al. (2012). Memory deficits in the transgenic rat model of Huntington's disease. [Research Support, Non-U.S. Gov't]. *Behavioural Brain Research, 227*(1), 194-198.

Zhu, X. R., Maskri, L., Herold, C., Bader, V., Stichel, C. C., Güntürkün, O., et al. (2007). Non-motor behavioural impairments in parkin-deficient mice. *European Journal of Neuroscience, 26*(7), 1902-1911.

Chapter 5

EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors

Nick P. van Goethem[1], Jos Prickaerts[1], Richard Chesworth, Gideon Shapiro, Frank G. Boess, Christoph Methfessel, Olga A. H. Reneerkens, Dorothy G. Flood, Dana Hilt, Maria Gawryl, Sonia Bertrand, Daniel Bertrand and Gerhard König
Neuropharmacology (2012), 62(2), 1099-1110

Abstract
EVP-6124, (*R*)-7-chloro-*N*-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide, is a novel partial agonist of α7 neuronal nicotinic acetylcholine receptors (nAChRs) that was evaluated here *in vitro* and *in vivo*. In binding and functional experiments, EVP-6124 showed selectivity for α7 nAChRs and did not activate or inhibit heteromeric α4β2 nAChRs. EVP-6124 had good brain penetration and an adequate exposure time. EVP-6124 (0.3 mg/kg, p.o.) significantly restored memory function in scopolamine-treated rats (0.1 mg/kg, i.p.) in an object recognition task (ORT). Although donepezil at 0.1 mg/kg, p.o. or EVP-6124 at 0.03 mg/kg, p.o. did not improve memory in this task, co-administration of these sub-efficacious doses fully restored memory. In a natural forgetting test, an ORT with a 24 h retention time, EVP-6124 improved memory at 0.3 mg/kg, p.o. This improvement was blocked by the selective α7 nAChR antagonist methyllycaconitine (0.3 mg/kg, i.p. or 10 μg, i.c.v.). In co-application experiments of EVP-6124 with acetylcholine, sustained exposure to EVP-6124 in functional investigations in oocytes caused desensitization at concentrations greater than 3 nM, while lower concentrations (0.3–1 nM) caused an increase in the acetylcholine-evoked response. These actions were interpreted as representing a co-agonist activity of EVP-6124 with acetylcholine on α7 nAChRs.

The concentrations of EVP-6124 that resulted in physiological potentiation were consistent with the free drug concentrations in brain that improved memory performance in the ORT. These data suggest that the selective partial agonist EVP-6124 improves memory performance by potentiating the acetylcholine response of α7 nAChRs and support new therapeutic strategies for the treatment of cognitive impairment.

Introduction

Nicotine has been shown to improve attention, learning, and memory through interaction with neuronal nAChRs (Levin et al., 2006). Several subtypes of nAChRs are expressed in the mammalian brain, each of them displaying distinct physiological and pharmacological properties. Functional nAChRs are assembled from five subunits around an axis of pseudosymmetry and can be composed of identical subunits (homopentamers) or different subunits (heteropentamers) (Dani and Bertrand, 2007). The α4 and β2 subunits are thought to form high affinity brain nAChRs, whereas the homopentameric α7 nAChR, which is expressed throughout the entire central nervous system, is less sensitive to ACh and nicotine (Dani and Bertrand, 2007; Albuquerque et al., 2009).

The α7 nAChRs are highly expressed in the hippocampus, a brain region that is very important for the formation of several types of memory. Hippocampal LTP, the sustained increase in the efficiency of synaptic transmission that is induced by multiple high frequency trains of electrical stimulation, is a potential cellular mechanism for learning and memory. In rat and mouse hippocampal slices, the partial α7 nAChR agonists GTS-21, SSR180711, and S 24795 improved LTP (Hunter et al., 1994; Biton et al., 2007; Lagostena et al., 2008). These effects were blocked by co-application of the α7 nAChR antagonist MLA and were absent in α7 nAChR knock-out mice (Lagostena et al., 2008). Furthermore, activation of the MAPK signaling pathway, with phosphorylation of ERK and CREB, is linked to the establishment of LTP and the formation of long term memory. The α7 nAChR agonists A-582941 and ABT-107 increased phosphorylation of ERK and CREB in the mouse brain (Bitner et al., 2007; Bitner et al., 2010).
Agonists of α7 nAChRs improved performance in learning and memory tasks (for review, see: Kem, 2000). GTS-21 improved inhibitory avoidance responding, one-way active avoidance, as well as performance in the Lashley III maze and the 17-arm radial maze in rats (Meyer et al., 1994; Arendash et al., 1995). In addition, GTS-21 facilitated performance in a delayed-matching-to-sample test in monkeys (Briggs et al., 1997). In clinical trials with healthy volunteers, GTS-21 improved attention, working memory, and episodic memory (Kitagawa et al., 2003). In a randomized double-blind crossover trial in nonsmoking subjects with schizophrenia, stably treated with antipsychotics, GTS-21 caused significant cognitive improvement on the Repeatable Battery for the Assessment of Neuropsychological Status total scale (Olincy et al., 2006). GTS-21 is a weak partial agonist of human α7 nAChRs and inhibits α4β2 nAChRs and 5-HT_3 receptors (Briggs et al., 1997). The more selective α7 nAChR agonist, AR-R17779, improved long-term win-shift acquisition in the eight-arm radial maze and social

recognition memory in rats (Levin et al., 1999; van Kampen et al., 2004). Improvements in social recognition, object recognition, and water maze performance were observed with several quinuclidine amide α7 nAChR agonists (Wishka et al., 2006; Boess et al., 2007; Hauser et al., 2009; Wallace et al., 2011), as well as with a number of other α7 nAChR agonists, including other types of quinuclidines (Pichat et al., 2007; Feuerbach et al., 2009; Sydserff et al., 2009; Bitner et al., 2010) and novel, structurally unrelated compounds (Bitner et al., 2007; Briggs et al., 2009; Roncarati et al., 2009). These cognitive enhancing effects were blocked by MLA (van Kampen et al., 2004; Boess et al., 2007; Pichat et al., 2007; Wallace et al., 2011), but were maintained by the co-infusion of donepezil, an AChEI (Bitner et al., 2010).

In this work, we examined the physiological and pharmacological properties of a novel and selective quinuclidine amide α7 nAChR agonist, EVP-6124, and its effects on memory in the ORT. Memory loss was either pharmacologically induced (i.e. disruption of the cholinergic system by administration of the muscarinic antagonist scopolamine) or was natural (i.e. a 24 h retention interval). Furthermore, EVP-6124 was co-administered with MLA (i.p. or i.c.v.) to investigate whether the pro-cognitive effects of EVP-6124 could be antagonized. Since α7 nAChR agonists have been suggested for the treatment of AD, and AD patients are often treated with an AChEI, the potential beneficial interaction between AChEIs and EVP-6124 was investigated in the present study at the behavioral level in rodents and at the electrophysiological level in oocytes expressing human α7 nAChRs.

Material and methods

Reagents

EVP-6124 was synthesized by Bayer Healthcare AG (Wuppertal-Elberfeld, Germany) and Ricerca Biosciences (Concord, OH). MLA and scopolamine hydrobromide were obtained from Research Biochemicals International/Sigma-Aldrich (Deisenhofen, Germany). Donepezil was a generous gift from Solvay Pharmaceuticals (Weesp, The Netherlands). Reagents for binding and electrophysiological studies were from Sigma-Aldrich. Reagents used in pharmacokinetic studies were from VWR International, Ltd. (Poole, Dorset, UK).

Animals

All animal experiments were approved by local ethical committees, followed the principles of laboratory animal care, and were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), or the Guide

for the Care and Use of Laboratory Animals from the U.S. Department of the Health and Human Services. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to use alternatives to *in vivo* methods where possible.

Selectivity profiling

Binding or activity of EVP-6124 was measured at 10 µM in a selectivity panel according to standard validated protocols under conditions defined by the contractor (MDS Pharma Services, Taipei, Taiwan; http://www.mdsps.com). Reference standards were run as an integral part of each assay to ensure the validity of the results.

For the 5-HT_{2A} receptor binding assay, membranes were prepared from HEK293 cells expressing the human recombinant 5-HT_{2A} receptor. For 5-HT_{2B} and 5-HT_{2C} receptor binding assays, membranes were prepared from CHO cells expressing the human recombinant 5-HT_{2B} or 5-HT_{2C} receptor. Affinity was determined by incubating different concentrations of EVP-6124 in binding buffer for 1 h. For 5-HT_{2A} binding, the incubation was at 22°C in the presence of 0.5 nM [3H]-ketanserin; for 5-HT_{2B}, at 22°C in the presence of 2 nM [^{3}H]-mesulergine; and for 5-HT_{2C}, at 37°C in the presence of 1 nM [^{3}H]-mesulergine. Nonspecific binding was determined in the presence of 1 µM ketanserin, 10 µM mesulergine, or 10 µM RS-102221 for 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C}, respectively. All measurements were performed in triplicate. EVP-6124 was also tested in the 5-HT_{2B} rat gastric fundus tissue response assay according to standard protocols under conditions defined by the contractor (MDS Pharma Services). Briefly, inhibition of α-methyl serotonin-induced contraction was isometrically measured. All measurements were performed in duplicate.

Binding assays

Brains from male Sprague-Dawley rats were rapidly removed and placed in ice cold homogenization buffer (10% w/v 0.32 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% w/v NaN_3, pH 7.4, 4°C). Brains were homogenized at 600 rpm in a Potter glass Teflon® homogenizer. The resulting suspension was centrifuged (1000 x g, 4°C, 10 min) and the supernatant was collected. The pellet was resuspended in homogenization buffer (20% w/v) and the suspension was recentrifuged (1000 x g, 4°C, 10 min). Both supernatants were combined and centrifuged (15 000 x g, 4°C, 30 min). The resulting pellet (P2 fraction) was resuspended in binding buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$, pH 7.4) and centrifuged (15 000 x g, 4°C, 30 min). The resuspension and centrifugation were repeated once.

For the α7 nAChR assay, the final pellet was resuspended in binding buffer and incubated in a final volume of 250 µl (0.2 mg membrane protein per assay) in the presence of 2 nM [^{3}H]-MLA, 0.1% w/v BSA and different concentrations of the test substance for 2 h at 25°C. Nonspecific binding was determined in the presence of 10 µM MLA. The incubation was terminated by the addition of 4 ml PBS (20 mM Na_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4, 4°C) and rapid filtration using a cell harvester (Brandel, Inc., Gaithersburg, MD) and type A/E glass fiber filters (Gelman Sciences, Inc., Ann Arbor, MI), pretreated for 3 h with 0.3% v/v polyethylenimine. Filters were washed twice with 4 ml PBS (4°C) and bound [^{3}H]-MLA was determined by scintillation counting. All measurements were performed in triplicate.

To determine the affinity of the compound at rat brain α4β2 nAChRs, different concentrations of EVP-6124 were incubated in a final volume of 250 µl binding buffer for 2 h at 4°C in the presence of 2 nM [^{3}H]-cytisine. Nonspecific binding was determined in the presence of 10 µM nicotine. All measurements were performed in triplicate.

The 5-HT_3 receptor is a homologue of the α7 nAChR and is often potently antagonized by α7 nAChR agonists (Briggs et al., 1997; Bodnar et al., 2005; Wishka et al., 2006; Sydserff et al., 2009; Wallace et al., 2011). For 5-HT_3 receptor binding assays, membranes from HEK293 cells expressing human recombinant 5-HT_3 receptor (RB-HS3, Receptor Biology, Inc., Beltsville, MD) were diluted according to manufacturer's instructions in incubation buffer (50 mM Tris-base, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% ascorbic acid, 10 µM pargyline) and incubated in a volume of 200 µl (membrane protein concentration: 3 µg per assay) for 60 min at 21°C in the presence of 0.5 nM of the selective 5-HT_3 receptor radioligand [^{3}H]-GR65630 and different concentrations of EVP-6124. Nonspecific binding was determined in the presence of 100 µM 5-HT. The incubation was terminated by filtration through type A/E glass fiber filters (Gelman Sciences, Inc.) or GF/B filters (Whatman, Maidstone, UK) that were pretreated for at least 1 h with 0.3% v/v polyethylenimine. Filters were washed three times with 3 ml buffer (50 mM Tris-HCl, pH 7.4; 4°C) and bound radioactivity was determined by scintillation counting. All measurements were performed in triplicate.

The IC_{50} values were determined from plots of binding activity versus log compound concentration using a sigmoidal curve fit (Prism, v. 2.0, GraphPad

Software Inc., San Diego, CA). The dissociation constants K_i of test compounds were determined from their IC_{50} values, the dissociation constants K_D, and the concentrations L of [^{3}H]-MLA, [^{3}H]-cytisine or [^{3}H]-GR65630 as appropriate, using the equation $K_i = IC_{50} / (1+L/K_D)$.

Electrophysiological recordings

Experiments were carried out with human α7 nAChRs expressed in *Xenopus laevis* oocytes. Oocytes were prepared, injected with cDNA encoding α7 nAChR subunits, and recorded using standard procedures (Hogg et al., 2008). Additional studies were carried out with rat α3β4, α4β2, and muscle α1β1γδ nAChRs expressed in oocytes. Briefly, ovaries were harvested from *Xenopus laevis* females that were deeply anesthetized by cooling at 4°C and with tricaine mesylate (3-aminobenzoic acid ethyl ester, methane sulfonate salt, 150 mg/l). Small pieces of ovary were isolated in sterile Barth solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4.7H_2O$, 0.33 mM $Ca(NO_3)_2.4H_2O$, and 0.41 mM $CaCl_2.6H_2O$, pH 7.4) and supplemented with 20 µg/ml kanamycin, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Injections of cDNAs encoding for the receptors were performed in at least one hundred oocytes using an automated injection device (Roboinject, Multi Channel Systems, Reutlingen, Germany); and receptor expression was examined at least two days later. Oocytes were impaled with two electrodes filled with 3 M KCl, and their membrane potentials were maintained at -80 mV throughout the experiment. All recordings were performed at 18°C and cells were superfused with OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM $CaCl_2.2H_2O$, and 1 mM $MgCl_2.6H_2O$, pH 7.4). Currents were recorded using an automated process equipped with standard two-electrode voltage-clamp configuration (HiClamp, Multi Channel Systems). Data were captured and analyzed using Matlab (Mathworks, Inc., Natick, MA) or Excel (Microsoft, Redmond, WA) software. ACh and EVP-6124 were prepared as concentrated stock solutions in water and then diluted in the recording medium to obtain the desired test concentrations. All experiments were carried out using three or more cells.

Object recognition task

Animals

In the dose-response experiment assessing the effect of EVP-6124 on a scopolamine-induced deficit, twenty-four 2.5-month-old male Wistar rats (Harlan Laboratories, Inc., Horst, The Netherlands; average body weight: 329 g) were used. Each rat was tested with 4 treatments (including the control conditions). In the ORT combination study of EVP-6124 and donepezil, another

cohort of twenty-four 5-month-old male Wistar rats was used (Harlan; average body weight: 465 g), of which 23 animals were included in the final analysis due to the continuous escaping of one rat from the apparatus. All 23 rats received each treatment, i.e. were tested 5 times. For the experiments using natural forgetting (i.e. a 24 h retention interval) and MLA, twenty-four 2-month-old male Wistar rats were obtained from Charles River Laboratories International, Inc. (Sulzfeld, Germany). In the dose-response experiment, the rats were 2.5 months old (average body weight: 319 g). For the co-administration of EVP-6124 and MLA (i.p.), the rats were 3 months old (average body weight: 357 g). For the co-administration of EVP-6124 and MLA (i.c.v.), the rats were 4 months old (average body weight: 407 g). Across the 3 experiments, rats were tested a total of 5–10 times. The effects of EVP-6124 on memory consolidation in particular were investigated in the natural forgetting test in the ORT, using twenty-four 3-month-old Wistar rats (Harlan Laboratories, Inc.; average body weight: 357 g). All 24 rats received each treatment, i.e. each rat was tested 7 times in two experiments. All animals were housed individually, which improves ORT performance (Beck and Luine, 2002), in standard type III Makrolon cages on sawdust bedding. The animals were on a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00 h); and food and water were given *ad libitum*. The rats were housed and tested in the same room. A radio, playing softly provided background noise to mask noises in the room. All testing was performed between 9:00 and 18:00 h under low illumination (20 lux).

Object recognition memory task

The ORT was performed as described elsewhere (Ennaceur and Delcour, 1988; Prickaerts et al., 1997). The apparatus and objects are identical to those described previously (Rutten et al., 2007). The same four objects were used during adaptation and in the studies. For two weeks, the animals were handled daily and adapted to the test procedures. For two days, the rats were allowed to explore the apparatus with no objects present, twice for 3 min each day. Afterwards, animals were adapted to the testing sessions and treatments with saline injections (i.p., p.o., and i.c.v.), until stable discrimination performance was achieved at the 1 and 24 h retention intervals.

A testing session consisted of two trials (T1 and T2), each with a duration of 3 min. The times spent exploring each object during T1 and T2 were recorded manually with a personal computer. Object exploration was defined as directing the nose to the object at a distance of not more than 2 cm or touching the object with the nose. During T1, the apparatus contained two identical objects. After T1, the animal was returned to its home cage. After a retention interval of

1 h (scopolamine-induced short-term memory deficit) or 24 h (natural forgetting), the animal was returned to the apparatus for T2. In T2, one of the two familiar objects was replaced by a new object. All objects and locations were used in a balanced manner to exclude possible object and/or location preferences. To avoid olfactory cues, the objects were thoroughly cleaned with 70% ethanol after each trial. The testing order of treatments was determined randomly and several treatment groups were tested each day (n = 8 per treatment group per testing day). The experimenter was blind to the treatments. Because rats were retested with different compound doses, test sessions were scheduled to allow at least a two-day wash-out period.

Drug administration

EVP-6124 was prepared in deionized water at an injection volume of 2 ml/kg. Scopolamine was prepared in saline at an injection volume of 1 ml/kg, with doses based on the weight of the salt. Donepezil was dissolved in saline at an injection volume of 2 ml/kg. For i.p. administration, MLA was dissolved in deionized water at an injection volume of 1 ml/kg. MLA was dissolved in saline for i.c.v. administration.

ORT study designs

Dose-response effect of EVP-6124 on a scopolamine-induced deficit

Before testing EVP-6124, the effects of scopolamine alone at 0.03, 0.1, or 0.3 mg/kg, i.p. in the ORT were determined (n = 8 per treatment). Scopolamine (0.1 mg/kg, i.p.) injected 30 min before T1 resulted in a robust deficit at T2 when a 1 h interval was used (data not shown). The d2 index was not significantly different from the chance level of performance; and there were no changes in exploratory behavior for 0.1 mg/kg, i.p. of scopolamine compared with saline. Subsequently, the ability of EVP-6124 to reverse the memory impairment induced by 0.1 mg/kg of scopolamine was tested. First, scopolamine and then EVP-6124 (0.03, 0.1, 0.3, and 1.0 mg/kg, p.o.) were administered 30 min before T1. For the control treatments, animals received either deionized water (p.o.) plus saline (i.p.) or deionized water (p.o.) plus 0.1 mg/kg scopolamine (i.p.).

Effect of EVP-6124 and donepezil in combination on a scopolamine-induced deficit

The AChEI, donepezil, was used to elevate ACh levels and was tested in combination with EVP-6124. Sub-efficacious doses of EVP-6124 (0.03 mg/kg) and donepezil were administered p.o. A 1 h retention interval between T1 and T2 was used with the scopolamine-induced deficit (0.1 mg/kg, i.p.). In a previous study we found that 0.1 mg/kg of donepezil was the highest dose with no effect

on a scopolamine-induced memory deficit (data not shown), and hence was considered to be the sub-efficacious dose for this compound. The order of compound administration was scopolamine, EVP-6124, and donepezil, 30 min before T1.

Dose-response effect of EVP-6124 on natural forgetting
Untreated Wistar rats show no significant object memory after 24 h (Rutten et al., 2007). The effect of EVP-6124 on long-term memory was investigated using a 24 h interval between T1 and T2. EVP-6124 was tested at 0.1, 0.3, and 1.0 mg/kg, p.o., administered 30 min before T1.

Effect of EVP-6124 and an α7 nAChR antagonist on natural forgetting
In order to determine whether the pro-cognitive effects of EVP-6124 (0.3 mg/kg, p.o.) on natural forgetting could be antagonized, MLA was co-administered. MLA was administered 1 h before T1 and EVP-6124, 30 min before T1. The optimal dose of EVP-6124 in the 24 h retention interval study was 0.3 mg/kg, and was used in combination with 0.1, 0.3, and 1.0 mg/kg, i.p. of MLA.

An additional MLA experiment was conducted via i.c.v. administration in 21 cannulated rats to confirm that the action of EVP-6124 was mediated through centrally located α7 nAChRs. Rats were fully anesthetized with isoflurane (induction: 5% and maintenance: 2%), were fixed in a stereotaxic frame, and a cannula was placed above the left lateral ventricle at the following coordinates with reference to bregma: 0.8 mm posterior, 1.55 mm lateral and 3.8 mm ventral (Paxinos and Watson, 1996). The tip of the cannula ended 1.0 mm above the lateral ventricle. The cannula was affixed to the skull using acrylic dental cement and two small screws. Animals were allowed to recuperate for two weeks before the adaptation and drug testing procedures started.

An injection needle, that was 1.0 mm longer than the guide cannula, was inserted into the cannula to deliver MLA (3 or 10 µg, i.c.v.) into the lateral ventricle in an injection volume of 2 µl (1 µl/min), 4 min before T1. The needle was left in place for an additional minute to prevent the reflux of infused MLA. EVP-6124 (0.3 mg/kg, p.o.) was administrated 30 min before T1.

At the conclusion of testing, verification of the correct cannula placement was performed in two randomly selected animals. After injection of methylene blue, the animals were decapitated and the brains were rapidly removed and sliced with a razor blade. The presence of dye in the ventricular system verified the

correct placement of the cannula in both animals. No methylene blue was observed in the surrounding tissue.

Effect of EVP-6124 on memory consolidation in the natural forgetting ORT
In order to assess effects on memory consolidation in particular since administration of a drug before T1 can affect both acquisition and consolidation processes, EVP-6124 was tested using a 24 h interval between T1 and T2 in the natural forgetting ORT. EVP-6124 was administered immediately after T1 (0.3, 1.0, and 3.0 mg/kg, p.o.) to assess memory consolidation and was compared to administration of EVP-6124 2 h before T1 (0.1, 0.3, 1.0, and 3.0 mg/kg, p.o.).

Statistical analyses
The ORT provides measures for exploration time and discrimination (Prickaerts et al., 1997). The relative discrimination (d2) index, which is independent of exploratory activity, was calculated as d2 = (b – a) / (a + b), where the times spent exploring the familiar and new object during T2 were represented as 'a' and 'b', respectively. The d2 index ranged from -1 to 1, with -1 or 1 indicating complete preference for the familiar or novel objects, respectively and 0 signifying no preference for either object.

One-sample *t*-tests were performed for each treatment to assess whether the d2 index significantly differed from zero, the chance level of performance. Effects between the treatments were assessed by either one-way or repeated measures ANOVAs. In the combination study of EVP-6124 and donepezil, all 23 animals received each treatment (within-subjects design), and a repeated measures ANOVA was used. In the other studies, different subsets of animals were used for each treatment and between-subjects, one-way ANOVAs were performed. When the overall ANOVA was significant, *post hoc* Bonferroni *t*-tests (all pairwise comparisons) were used. An α level of 0.05 was considered significant. Of note, overall the total object exploration times in T1 and T2 did not differ after treatment with EVP-6124, MLA, scopolamine, or donepezil in any of the tests (data not shown), indicating that the compounds did not affect activity and/or exploratory behavior per se. In the consolidation study, in which rats were treated after T1, there was an incidental finding of differences between the groups for exploration times during T1 (data not shown).

Pharmacokinetics and determination of plasma protein binding
Pharmacokinetics
Male Wistar rats (Charles River Labs, Wilmington, MA; approximately 270 g, n = 60) were treated with EVP-6124. EVP-6124 was prepared in 0.25% aqueous

methylcellulose (Sigma-Aldrich, St. Louis, MO) at a volume of 1 ml/kg for p.o. administration. Animals were sacrificed with CO_2 at 1, 2, 4, or 8 h after dosing; blood was collected via cardiac puncture and brains were removed and frozen. Blood was collected in lithium heparin tubes and centrifuged; and plasma was removed and stored frozen until analysis. Brains were homogenized in PBS (0.1 M, pH 7.4), centrifuged at 15,000 rpm for 10 min at 4°C, and the supernatant collected and stored frozen until analysis.

Plasma protein binding

The rat plasma protein binding assays were performed using an equilibrium dialysis method adapted from Ponganis and Stanski (1985). Each dialysis cell (Dianorm, Munich, Germany) consisted of two compartments separated by a cellulose semipermeable membrane with a molecular weight cut off of 5000 Da. The membrane was rehydrated before use in deionized water followed by the dialysis buffer. Plasma (Harlan Sera-Lab Limited, Loughborough, UK) was heated to 37°C and adjusted to pH 7.4 before use. Five micromolar EVP-6124 solutions were prepared in isotonic phosphate buffer and species-specific plasma (final dimethyl sulfoxide concentration of 0.5%). The plasma was introduced to one side of the membrane, and dialysis buffer to the plasma-free, other side. Incubations were performed for 2 h in duplicate. The dialysis cells were mounted and rotated in a drive unit to ensure that a uniform equilibrium was obtained. The equilibrium was temperature controlled by immersing the drive unit in a water bath at 37°C. At the end of the equilibration time the cells were emptied.

Bioanalytical methods

Following protein precipitation by the addition of methanol containing an internal standard, the samples were centrifuged and analyzed by LC-MS/MS. The LC system consisted of an Agilent HP 1100 binary LC pump (Agilent Technologies, UK, Ltd., Stockport, Cheshire, UK) and CTC Analytics HTS autosampler (Presearch Ltd, Hitchin, Herts, UK). This system used an Atlantis C18 3 µm column (10 x 2.1 mm) (Waters Ltd, Elstree, Herts, UK) maintained at 40°C running a solvent gradient of 10 mM ammonium formate in deionized water containing 0.1% formic acid (Eluent A) and 10 mM ammonium formate in methanol containing 0.1% formic acid (Eluent B). Solvent composition was maintained at 100% A for 0.1 min following injection of each sample. A linear LC gradient was then employed reaching 5% Eluent A at 1.5 min (held for 0.3 min), and 100% Eluent A from 1.85 min until the end of the run. The flow rate was 0.5 ml/min. Triple quadrupole MS/MS analyses were performed using a Quattro Micro (Waters Ltd). Analyte detection used parent and daughter ion masses

identified by an automated optimization process using Masslynx software with the Quanlynx application manager (Waters Ltd). The plasma and brain samples from rats were quantified using standard curves prepared in plasma or brain homogenate, respectively. The dialysis cell samples from the protein containing compartment were quantified using calibration standards prepared in plasma and the protein free compartments were quantified using calibration standards prepared in dialysis buffer. The fraction unbound was determined using the formula: fu = 1-((PC-PF)/PC), where PC = sample concentration in protein-containing side, PF = sample concentration in protein free-side.

Results

Selectivity profile of EVP-6124

EVP-6124 is a novel synthetic molecule (Fig. 1) that was found to bind with high affinity to α7 nAChRs in rat brain membranes and to displace radioactive ligands such as the snake toxin α-bungarotoxin and MLA, two ligands that are specific for α7 nAChRs. EVP-6124 displaced [^{3}H]-MLA (K_i = 9.98 nM, pIC_{50} = 7.65 ± 0.06, n = 3; Fig. 2A) and [^{125}I]-α-bungarotoxin (K_i = 4.33 nM, pIC_{50} = 8.07 ± 0.04, n = 3; data not shown). EVP-6124 was approximately 300 fold more potent than the natural agonist ACh (K_i = 3 μM), measured in binding assays using [^{3}H]-MLA (data not shown).

The specificity of EVP-6124 for α7 nAChRs was confirmed by the absence of displacement of [^{3}H]-cytisine from α4β2 nAChRs by 10 μM EVP-6124, suggesting that the affinity of EVP-6124 for these heteromeric receptors was at least 1000 fold lower than the affinity of nicotine (K_i = 8 nM) for the α4β2 nAChRs labeled by [^{3}H]-cytisine. The selectivity of EVP-6124 was further examined using a panel of more than 60 molecular targets, including peptide and non-peptide receptors, ion channels, and amine transporters (Table 1). No appreciable interaction was found with any of the examined targets in this panel, other than at the 5-HT_3 receptor subtype. EVP-6124 inhibited the 5-HT_3 receptor by 51% at 10 nM, the lowest concentration tested (data not shown). Evaluation of the human 5-HT_{2B} receptor expressed in CHO cells demonstrated displacement of [^{3}H]-mesulergine (K_i = 14 nM) and only antagonist activity in the rat gastric fundus assay at an IC_{50} of 16 μM.

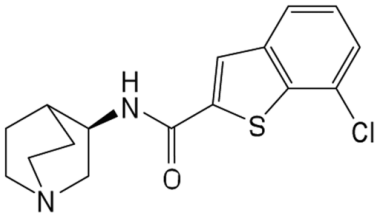


Figure 1. Representation of the chemical structure of EVP-6124, (*R*)-7-chloro-*N*-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide.

Table 1. *In vitro* binding or activity of EVP-6124

Target	Percent inhibition (10 µM)	Target	Percent inhibition (10 µM)
Adenosine A_1 (*h*)	-7	Neuropeptide Y Y_1 (*h*)	-4
Adenosine A_{2A} (*h*)	12	Neuropeptide Y Y_2 (*h*)	-3
Adenosine A_3 (*h*)	10	Nicotinic ACh (*h*)	34
Adrenergic α_{1A} (*r*)	14	Nicotinic Ach, α7 (*r*)	99
Adrenergic α_{1B} (*r*)	46	Nicotinic Ach bungarotoxin-sensitive neuromuscular (*h*)	15
Adrenergic α_{1D} (*h*)	39	Opiate δ (*h*)	0
Adrenergic α_{2A} (*h*)	46	Opiate κ (*h*)	11
Adrenergic β_1 (*h*)	24	Opiate µ (*h*)	33
Adrenergic β_2 (*h*)	6	Platelet activating factor	-2
Bradykinin B_1 (*h*)	10	Prostanoid EP_4 (*h*)	-4
Bradykinin B_2 (*h*)	12	Purinergic P_{2x} (*rb*)	-5
Dopamine D_1 (*h*)	37	Purinergic P_{2Y} (*r*)	5
Dopamine D_{2S} (*h*)	36	5-HT_{1A} (*h*)	31
Dopamine D_3 (*h*)	16	5-HT_3 (*h*)	99
Dopamine $D_{4.2}$ (*h*)	14	Sigma σ_1 (*h*)	62
Endothelin ET_A (*h*)	4	Sigma σ_2 (*r*)	54
Endothelin ET_B (*h*)	-11	Tachykinin NK_1 (*h*)	-24
Epidermal growth factor (EGF) (*h*)	6	Testosterone (*r*)	2
Estrogen ERα (*h*)	4	Thyroid hormone (*r*)	-16
$GABA_A$, agonist site (*r*)	14	Calcium channel L-type, benzothiazepine (*r*)	13
$GABA_A$, central benzodiazepine (*r*)	1	Calcium channel L-type, dihydropyridine (*r*)	24
$GABA_{B1A}$ (*h*)	-18	Calcium channel N-type (*r*)	4
Glucocorticoid (*h*)	0	Potassium channel [K_{ATP}] (*h*)	-1
Glutamate, kainate (*r*)	-8	Sodium channel, site 2 (*r*)	59
Glutamate, NMDA, agonism (*r*)	5	Dopamine transporter (*h*)	29
Glutamate, NMDA, glycine (*r*)	-6	GABA transporter (*r*)	18
Glutamate, NMDA, phencyclidine (*r*)	0	Norepinephrine transporter (*h*)	53
Histamine H_1 (*h*)	24	5-HT transporter (*h*)	2
Histamine H_2 (*h*)	15	Phorbol ester (*m*)	-7
Histamine H_3 (*h*)	-6	Rolipram (*r*)	2
Imidazoline I_2, central (*r*)	21	CYP450 1A2 (*h*)	7
Interleukin IL-1 (*m*)	-16	CYP450 2C19 (*h*)	5
Leukotriene $CysLT_1$ (*h*)	4	CYP450 2C9 (*h*)	3
Melotonin MT_1 (*h*)	5	CYP450 2D6 (*h*)	-20
Muscarinic M_1 (*h*)	19	CYP450 3A4 (*h*)	8
Muscarinic M_2 (*h*)	5		
Muscarinic M_3 (*h*)	7		

h, human; *m*, mouse; *r*, rat; *rb*, rabbit

Determination of the activity of EVP-6124

In electrophysiological studies on *Xenopus* oocytes expressing human α7 nAChRs, brief pulses of EVP-6124 produced strong inward currents relative to 50 µM ACh (Fig. 2B). In double-logarithmic format, the straight-line fit of the dose-response curve yielded an EC_{50} of 0.16 µM and a slope of 1.6 for EVP-6124. Relative to 1280 µM ACh, EVP-6124 acted as a partial agonist, with maximum current amplitude of 42 ± 3% (Fig. 2C). Typical EVP-6124-evoked currents (Fig. 2C, inset), and the corresponding concentration activation curve (Fig. 2C) were generated by pulsing EVP-6124 every minute at increasing concentrations up to 30 µM. These data were readily fitted with a single Hill equation with an EC_{50} of 0.39 ± 0.07 µM and a Hill coefficient of 1.45 ± 0.11 (n = 8). The reduction in the amplitude of the inward current at 10 and 30 µM, after increasing concentrations of EVP-6124 suggested that the compound desensitized α7 nAChRs. To better assess the agonist activity of EVP-6124 without desensitization, cells expressing α7 nAChRs were first challenged with an ACh test pulse (1280 µM) and then with a single pulse of 30 µM EVP-6124 (Fig. 2D). Currents evoked by 30 µM reached 82 ± 7% of the current evoked by ACh (Fig. 2E), which was considerably larger than the 42% observed in Fig. 2C. Additionally, single pulses of 30 µM EVP-6124 demonstrated that the compound acted as a partial but potent agonist at α7 nAChRs (Fig. 2D-E).

Selectivity of EVP-6124 was further confirmed using electrophysiological characterization of the rat α3β4, α4β2, and muscle α1β1γδ receptors. There was no detectable agonist activity at concentrations up to 100 µM. Antagonist activity was, however, observed at the rat α3β4 receptor with an IC_{50} of 16 µM (data not shown).

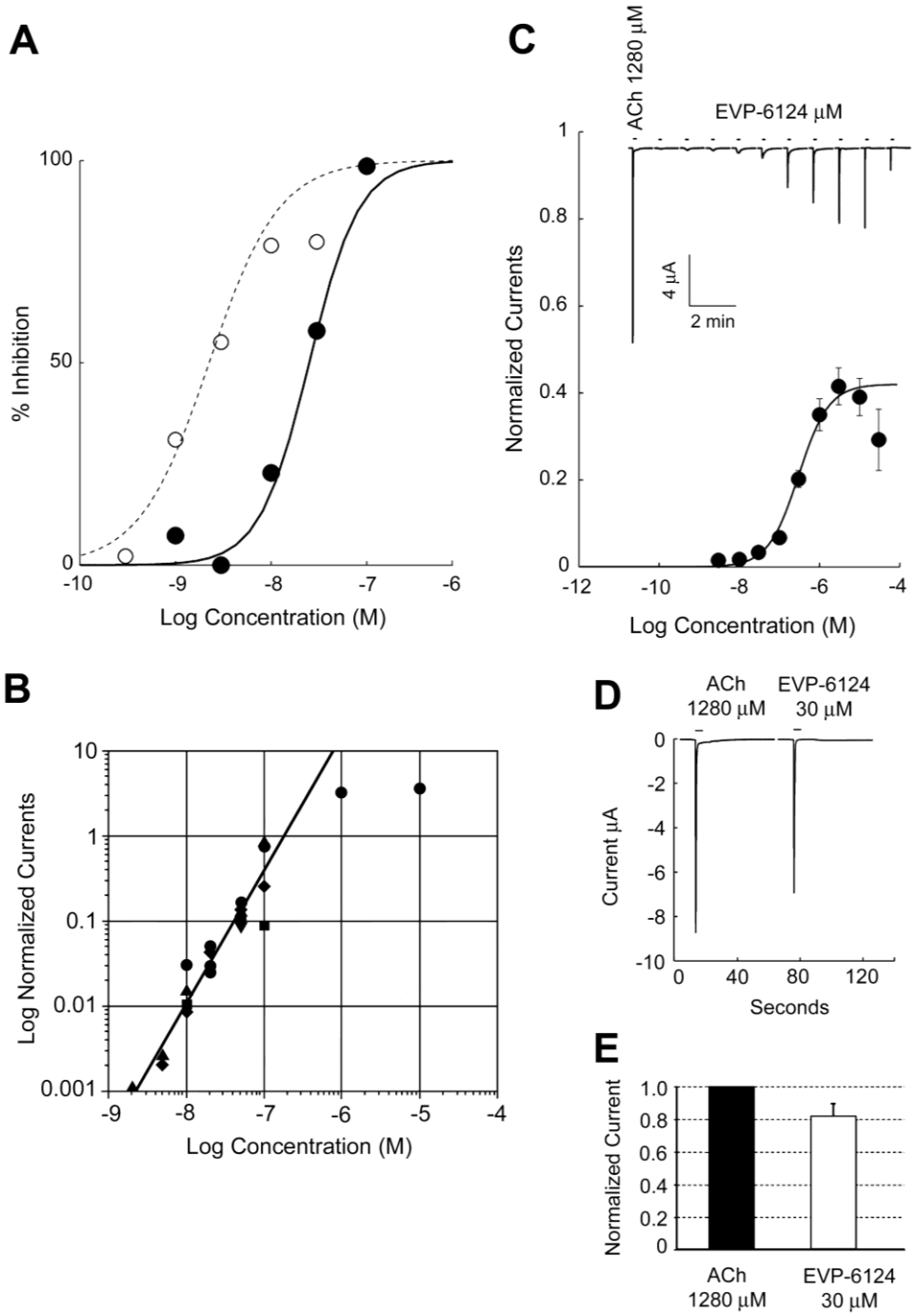

A
100
50
0
% Inhibition
-10
-9
-8
-7
-6
Log Concentration (M)
C
ACh 1280 µM
EVP-6124 µM
1
0.8
0.6
0.4
0.2
0
Normalized Currents
4 µA
2 min
-12
-10
-8
-6
-4
Log Concentration (M)
B
10
1
0.1
0.01
0.001
Log Normalized Currents
-9
-8
-7
-6
-5
-4
Log Concentration (M)
D
ACh
1280 µM
EVP-6124
30 µM
0
-2
-4
-6
-8
-10
Current µA
0
40
80
120
Seconds
E
1.0
0.8
0.6
0.4
0.2
0
Normalized Current
ACh
1280 µM
EVP-6124
30 µM

Figure 2. A) EVP-6124 efficiently displaced [^{3}H]-MLA binding in rat brain homogenates, yielding a K_i of 9.98 nM (filled symbols and continuous line). As a positive control, open symbols and dashed line indicate the displacement of [^{3}H]-MLA caused by α-bungarotoxin with a K_i of 1.14 nM. **B)** EVP-6124 acted as an agonist of human α7 nAChRs expressed in *Xenopus* oocytes. The dose response curve for peak current was plotted in double-logarithmic format, normalized to the current evoked by 50 µM ACh. The EC_{50} was 0.16 µM and the slope was 1.6. **C)** EVP-6124 acted as a partial agonist at human α7 nAChRs expressed in *Xenopus* oocytes. Inset, typical currents evoked by a brief pulse of ACh (1280 µM), a pulse with no compounds, and a series of pulses of increasing concentrations of EVP-6124 from 0.003–30 µM are shown. Detectable responses were seen at concentrations greater than 0.03 µM. The continuous line on the concentration activation curve is the best fit obtained with the Hill equation, an EC_{50} of 0.39 ± 0.07 µM, and a Hill coefficient of 1.45 ± 0.11. Bars indicate SEM (n = 8). **D)** EVP-6124 acted as a potent partial agonist at human α7 nAChRs. Typical currents recorded in an oocyte expressing human α7 nAChRs in response to 1280 µM ACh and to a single pulse of 30 µM EVP-6124. Bars above the traces indicate the timing of the drug application. **E)** Histogram of the currents normalized versus the ACh response for experiments as described in **D**. n = 8, bars indicate SEM. A comparison of **C** with **D-E** shows that repeated application of EVP-6124 desensitized human α7 nAChRs.

Effects of EVP-6124 on memory in the object recognition task

EVP-6124 reverses a scopolamine-induced deficit

Comparisons between treatments indicated differences for the d2 indices ($F_{5,90}$ = 15.60, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly higher after treatment with saline and water vehicles (without a scopolamine deficit) and in the presence of 0.3 and 1.0 mg/kg of EVP-6124 (with a scopolamine deficit) than after treatment with scopolamine alone (Fig. 3A). One-sample *t*-tests showed significant differences from chance performance for the d2 indices at 0.1, 0.3, and 1.0 mg/kg of EVP-6124 and 0.1 mg/kg of scopolamine, indicating recognition of the familiar object and reversal of the scopolamine-induced deficit (Fig. 3A). After treatment with both vehicles, the d2 index was also significantly different from chance performance.

Sub-efficacious doses of EVP-6124 and an AChEI (donepezil) reverse a scopolamine-induced deficit

The repeated measures ANOVA revealed differences between the treatments for the d2 indices ($F_{4, 88}$ = 8.18, $p < 0.001$). *Post hoc* analysis revealed that the d2 indices were significantly higher for the vehicle and the EVP-6124 and donepezil combined treatments, when compared with scopolamine alone (Fig. 3B). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the vehicle and for the combination of EVP-6124 and donepezil (Fig. 3B). This was in contrast to the other treatments, which showed no significant differences from chance performance.

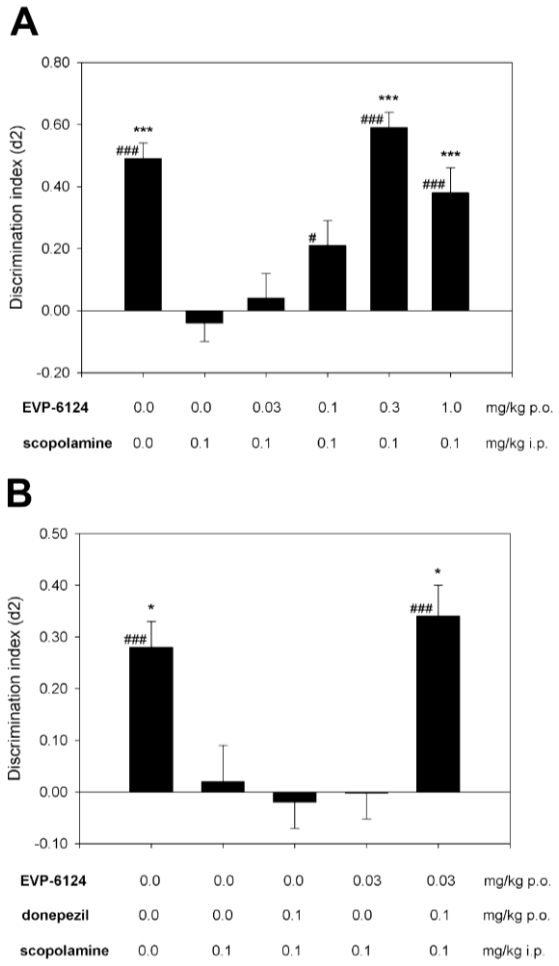


Figure 3. Scopolamine-induced short-term memory deficit in an object recognition task. All drugs were administered 30 min before T1. **A)** Effects of EVP-6124 (p.o.) on the discrimination index (d2) using scopolamine treatment (0.1 mg/kg, i.p.) to impair memory and a 1 h interval in 2.5-month-old male Wistar rats (mean + SEM). When compared to scopolamine alone, EVP-6124 (0.3 and 1.0 mg/kg) reversed the short-term memory impairment induced by treatment with scopolamine. Differences from scopolamine alone: ***$p < 0.001$. Differences from zero (chance performance): $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$. n = 24 per treatment for vehicle and scopolamine alone, and 12 per treatment with EVP-6124. **B)** The effects of donepezil (p.o.) and EVP-6124 (p.o.) on the d2 index in an ORT using a 1 h interval, after scopolamine treatment (i.p.) in 5-month-old male Wistar rats (mean + SEM). When compared to scopolamine alone, EVP-6124 and donepezil combined reversed the scopolamine-induced memory deficit. Differences from scopolamine alone: *$p < 0.05$. Differences from chance performance: $^{\#\#\#}p < 0.001$. n = 23 per treatment.

EVP-6124 prevents natural forgetting

Comparison between treatments indicated differences for the d2 indices ($F_{3,60}$ = 3.62, $p < 0.05$). *Post hoc* analysis showed that the d2 index was significantly higher for the 0.3 mg/kg EVP-6124 treatment when compared with the vehicle (Fig. 4A). One-sample *t*-tests showed significant differences from chance performance for the d2 indices at EVP-6124 doses of 0.1, 0.3, and 1.0 mg/kg, indicating recognition of the familiar object and prevention of natural forgetting (Fig. 4A).

MLA antagonism of the memory enhancing effects of EVP-6124 on natural forgetting

For the i.p. MLA study, comparison between treatments indicated differences for the d2 indices ($F_{4,75}$ = 16.94, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly lower after treatment with only the vehicles and with 0.3 and 1.0 mg/kg of MLA in combination with 0.3 mg/kg of EVP-6124 than after treatment with 0.3 mg/kg of EVP-6124 alone (Fig. 4B). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the combination of 0.3 mg/kg of EVP-6124 with vehicle or 0.1 mg/kg of MLA, indicating prevention of natural forgetting (Fig. 4B).

For the i.c.v. MLA study, comparison between treatments indicated differences for the d2 indices ($F_{3,60}$ = 10.17, $p < 0.001$). *Post hoc* analysis showed that the d2 indices were significantly lower after treatment with only the vehicles and with 10 µg of MLA in combination with 0.3 mg/kg of EVP-6124 than after treatment with 0.3 mg/kg of EVP-6124 alone (Fig. 4C). One-sample *t*-tests showed significant differences from chance performance for the d2 indices for the combination of 0.3 mg/kg of EVP-6124 with vehicle or 3 µg of MLA, indicating prevention of natural forgetting (Fig. 4C).

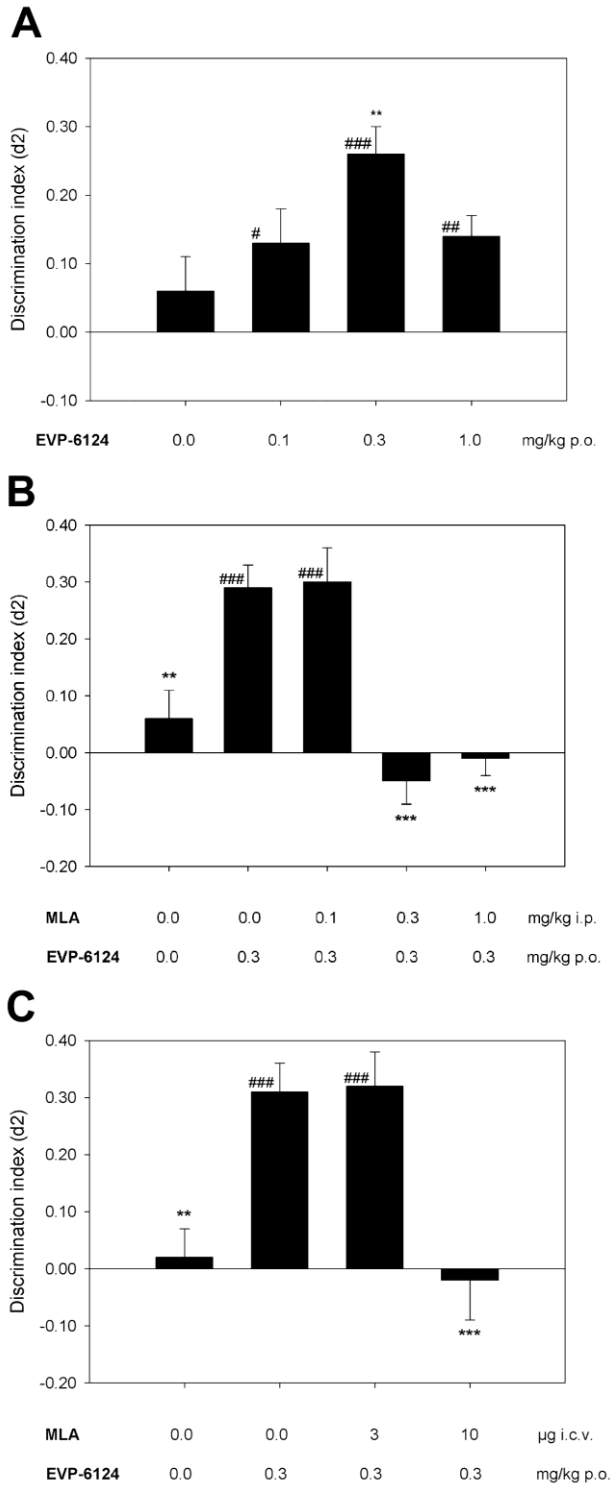


Figure 4. Natural forgetting in an object recognition task**.** EVP-6124 was always administered 30 min before T1. **A)** Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 2.5-month-old male Wistar rats (mean + SEM). When compared to vehicle, EVP-6124 (0.3 mg/kg) prevented loss of long-term memory. Differences from vehicle: $^{**}p < 0.01$. Differences from chance performance: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$. n = 16 per treatment. **B)** The effects of co-administration of 0.1–1 mg/kg of MLA (i.p.) 1 h before T1 and 0.3 mg/kg of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to the administration of 0.3 mg/kg of EVP-6124 alone, 0.3 and 1.0 mg/kg of MLA significantly antagonized the pro-cognitive effect of 0.3 mg/kg of EVP-6124. Differences from 0.3 mg/kg EVP-6124 alone: $^{**}p < 0.01$; $^{***}p < 0.001$. Differences from chance performance: $^{\#\#\#}p < 0.001$. n = 16 per treatment. **C)** The effects of co-administration of 3 and 10 µg of MLA (i.c.v.) 4 min before T1 and 0.3 mg/kg of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 4-month-old male Wistar rats (mean + SEM). When compared to the administration of 0.3 mg/kg of EVP-6124 alone, 10 µg of MLA significantly blocked the pro-cognitive effect of 0.3 mg/kg of EVP-6124. Differences from 0.3 mg/kg EVP-6124 alone: $^{**}p < 0.01$; $^{***}p < 0.001$. Differences from chance performance: $^{\#\#\#}p < 0.001$. n = 16 per treatment.

Effects of EVP-6124 on consolidation in the natural forgetting ORT

A vehicle treatment was not used in these studies, since in the previous experiments vehicle administered before T1 produced natural forgetting. Thus, no ANOVAs were performed. When EVP-6124 was administered before T1, the one-sample *t*-tests showed significant differences from chance performance for the d2 indices for 0.3, 1, and 3 mg/kg of EVP-6124 (Fig. 5A). When EVP-6124 was administered immediately after T1, to selectively assess the effect on memory consolidation, the one-sample *t*-tests showed significant differences from chance performance for the d2 indices for EVP-6124 at 1 and 3 mg/kg (Fig. 5B).

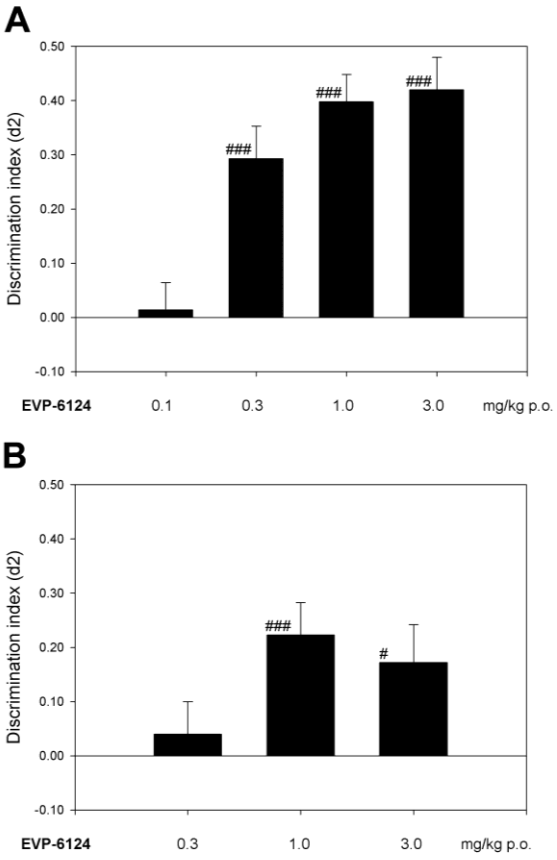


Figure 5. Effect of EVP-6124 on memory consolidation in the natural forgetting ORT **A)** Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to chance level, EVP-6124 (0.3 - 3 mg/kg, p.o.) administered 2 h before T1 improved memory. Differences from chance performance: $^{###}p < 0.001$. n = 24 per treatment. **B)** Effects of EVP-6124 (p.o.) on the d2 index using a 24 h retention interval in 3-month-old male Wistar rats (mean + SEM). When compared to chance performance, EVP-6124 (1 and 3 mg/kg) administered immediately after T1 improved memory consolidation. Differences from chance performance: $^{#}p < 0.05$; $^{###}p < 0.001$. n = 24 per treatment.

Pharmacokinetics and brain penetration of EVP-6124 in rats

EVP-6124 was found to bind moderately to rat plasma proteins with a mean fu of 0.11 ± 0.01 (mean ± SD) or 11%. Over a range of 0.1–30 mg/kg, p.o., EVP-6124 demonstrated proportional dose escalation. Table 2 shows the plasma and brain concentrations and B:P ratios of EVP-6124 after a single dose of 0.3 mg/kg, p.o., an efficacious dose in the ORT. T_{max} was at 4 h in plasma and 2 h brain, although the brain concentrations remained similar between 2 and 8 h. The B:P ratios were 1.7 to 5.1 between 1 and 8 h. Assuming that the free drug fraction in the brain was equal to or at least similar to the free drug fraction in the plasma, the corresponding brain free drug concentrations were between 0.14 and 0.24 nM up to 8 h after treatment with EVP-6124. The unbound concentration of EVP-6124 was assumed to reflect the concentration available for binding to brain α7 nAChRs.

Table 2. Total concentrations of EVP-6124 and (estimated free concentrations of EVP-6124) after a single dose of 0.3 mg/kg, p.o.

	Mean ± SEM		
Time (h)	**Plasma concentration (nM)**	**Brain concentration (nM)**	**Plasma to brain ratio**
1	0.71 ± 0.05 (0.078 ± 0.006)[1]	1.3 ± 0.2 (0.14 ± 0.02)	1.8
2	0.84 ± 0.13 (0.092 ± 0.014)	2.2 ± 0.2 (0.24 ± 0.03)	2.6
4	1.1 ± 0.1 (0.12 ± 0.01)	1.8 ± 0.5 (0.20 ± 0.05)	1.7
8	0.42 ± 0.11 (0.046 ± 0.012)	2.1 ± 0.5 (0.24 ± 0.05)	5.1

[1]Estimated free drug concentration = total drug concentration x fu, where fu = 0.11.
n = 5 per group

Low concentrations of EVP-6124 potentiate ACh-evoked currents

Estimation of the brain concentration of EVP-6124 attained during positive behavioral tests indicated that this compound was present in the sub- to low nanomolar range. Importantly, however, these concentrations were insufficient to activate or desensitize α7 nAChRs in electrophysiological studies (Fig. 2). To understand the possible mechanisms underlying the positive effects of EVP-6124 on cognition and memory, additional functional investigations were carried out.

The α7 nAChR desensitization profile caused by sustained application of EVP-6124 was determined by exposing oocytes expressing human α7 nAChRs to a succession of increasing EVP-6124 concentrations and measuring the response to fixed, low concentration ACh (40 μM) test pulses. Plotting the amplitude of the current evoked by ACh test pulses as a function of the logarithm of the concentration of EVP-6124 yielded a concentration inhibition curve with a sharp decline and an IC_{50} of 3 nM (Fig. 6A). This IC_{50} was in good agreement with the EVP-6124 K_i of 9.98 nM for displacement of [^{3}H]-MLA binding (Fig. 2A) and of 4.33 nM for displacement of [^{125}I]-α-bungarotoxin. At lower concentrations, the concentration inhibition curve for EVP-6124 showed an increase in the response to a pulse of ACh in the range of 0.3–1 nM. To assess the effect of EVP-6124 at potentiating (0.3 nM) and desensitizing (> 1 nM) concentrations on ACh-evoked responses over time, oocytes were exposed to a sustained concentration of EVP-6124 and their responses to 40 μM ACh test pulses were monitored at 2-min intervals (Fig. 6B). A marked and sustained increase (potentiation) in the ACh-evoked α7 nAChR response was observed at 0.3 nM of EVP-6124 (Fig. 6B, upper panel). At 3 nM of EVP-6124, an increase in the first ACh-evoked current was observed, followed by a rapid decline in the ACh-evoked α7 nAChR response, attributable to α7 nAChR desensitization (Fig. 6B, lower panel).

The effect of sustained application of EVP-6124 was then tested with ACh (40 μM) pulsed with a long time delay between pulses (Fig. 6C-D). Oocytes expressing α7 nAChRs were first tested with brief ACh pulses (40 μM, 5 sec) during a stabilization period (8 min) and then exposed for a prolonged period (20 min) to EVP-6124 at 0.3 nM. During the initial co-exposure to ACh and EVP-6124, the response amplitudes were larger than the response amplitudes for ACh alone, confirming the result in Fig. 6B, upper panel. Exposure to 0.3 nM EVP-6124, without pulses of ACh, did not evoke a sizeable current. The periodic test pulses of ACh, in the presence of 0.3 nM EVP-6124 in the medium, produced increased response amplitudes, larger than the amplitudes during the initial sustained co-application of ACh and EVP-6124. The minimal decline of the ACh-evoked current observed during washout of EVP-6124 suggested a slow off-rate of EVP-6124 from the receptors, which was consistent with the high affinity binding observed by the displacement of [^{3}H]-MLA and [^{125}I]-α-bungarotoxin from α7 nAChRs (Fig. 2A).

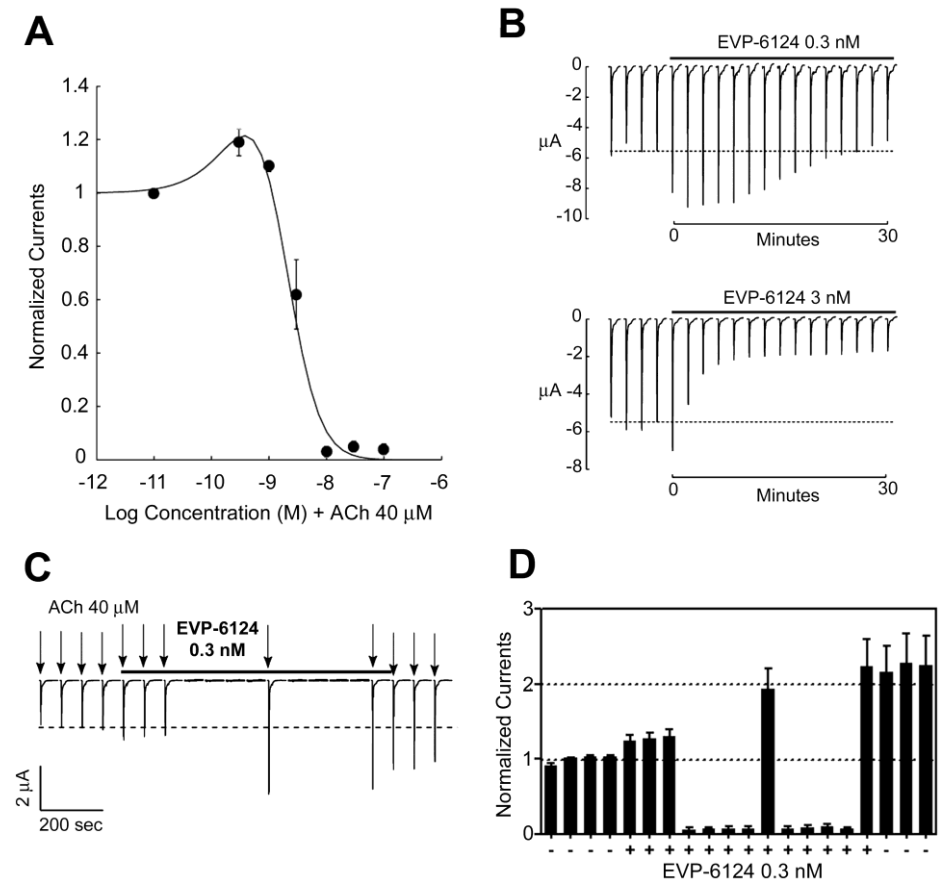


Figure 6. Low concentrations of EVP-6124 potentiate ACh-evoked currents. **A)** The response to a test pulse of ACh (40 µM) was measured during sustained exposure to EVP-6124 at the indicated logarithmic concentrations. The concentration inhibition curve for EVP-6124 showed an increase in the response to a pulse of ACh in the range of 0.3–1 nM. Higher concentrations of EVP-6124 yielded a concentration inhibition curve with a sharp decline and an IC_{50} of 3 nM. Filled circles are the mean of the normalized response ($n \geq 3$). Error bars are SEM. The curve is the best fit obtained with the dual binding site model (Cachelin and Rust, 1994; Zwart et al., 2000). **B)** Currents evoked by 40 µM ACh for 5 sec were measured at regular intervals of every 2 min, before and during a sustained exposure to EVP-6124 (0.3 and 3 nM). Bars above the traces indicate the timing of the sustained application of EVP-6124. EVP-6124 in the sub-nanomolar range potentiated ACh-evoked currents (upper panel), while EVP-6124 in the low nanomolar range reduced ACh-evoked currents (lower panel). **C)** α7 nAChRs were assessed by exposing the oocytes to brief ACh test pulses (arrows, 40 µM, 5 sec), first during a stabilization period of 8 min and then for a period of 20 min to EVP-6124 at 0.3 nM (indicated by the horizontal bar above the traces). Exposure to 0.3 nM EVP-6124 increased the ACh-evoked current without desensitizing the receptors. The magnitude of the increase in the ACh-evoked current was greater when the pulse was preceded by a rest period. **D)** Histogram of the currents (mean + SEM) normalized versus the initial responses to ACh alone for a series of experiments (n = 7) utilizing the protocol described in **C**.

Discussion

The identification of a high expression level of neuronal nAChRs in many brain areas, including the hippocampus and cortex, and more specifically of those containing homopentameric α7-subunits suggested that these receptors may play an important role in cognitive functions (Dani and Bertrand, 2007). This hypothesis was confirmed by the finding that specific agonists of α7 nAChRs improved memory performance in animals, as shown initially by the effects of molecules such as GTS-21 (reviewed in: Kem, 2000) and more recently by more selective and potent agonists.

Recently described α7 nAChRs agonists have demonstrated marked improvements in binding displacement of MLA or α-bungarotoxin, with K_i values in the range of 1–8.8 nM (Wishka et al., 2006; Hauser et al., 2009; Sydserff et al., 2009; Malysz et al., 2010; Wallace et al., 2011). EVP-6124 similarly displaced binding to the rat brain α7 nAChR with a K_i in the low nanomolar range (i.e. 9.98 nM for the displacement of [^{3}H]-MLA and 4.33 nM for [^{125}I]-α-bungarotoxin). Like EVP-6124, all of these potent agonists (TC-5619, ABT-107, AZD0328, RG3487, and PHA-543613) are quinuclidines. Earlier quinuclidines (e.g. PNU-282987, ABBF, and SSR180711), demonstrated less potency (K_i values of 22–62 nM) (Bodnar et al., 2005; Biton et al., 2007; Boess et al., 2007).

The selectivity of EVP-6124 for α7 nAChRs was demonstrated by the lack of effect on cytisine binding at α4β2 nAChRs. In *Xenopus* oocytes, EVP-6124 also did not activate heteromeric nAChRs, such as the α3β4, α4β2, and the embryonic muscle α1β1γδ subtypes. The selectivity for α7 nAChRs versus other nAChRs is characteristic of the other recently described compounds (Wishka et al., 2006; Hauser et al., 2009; Sydserff et al., 2009; Malysz et al., 2010; Wallace et al., 2011). EVP-6124 at a 10 μM concentration also lacked appreciable interactions with more than 60 molecular targets in a selectivity screening panel that included receptors, ion channels, and amine transporters. The antagonist activity of EVP-6124 at 5-HT_3 receptors, homologues of α7 nAChRs, is a common feature of some of the recently characterized compounds, such as AZD0328 and RG3487 (Sydserff et al., 2009; Wallace et al., 2011), and could prove beneficial in reducing the potential emetic effects of nicotinic agonists. In contrast, some of the quinuclidines achieved greater separation between the activities at α7 nAChRs and 5-HT_3 receptors (Wishka et al., 2006; Hauser et al., 2009; Malysz et al., 2010). Given the sub- to low nanomolar brain concentrations required for activation of α7 nAChRs, this greater separation may provide no therapeutic benefit in the clinic.

Functional assessment of EVP-6124 at α7 nAChRs revealed that the compound acted as a partial agonist and evoked up to 80% of the ACh-evoked current for single pulses of 30 μM. With application of ascending concentrations of EVP-6124, the maximum activity was 42% of ACh. Under similar experimental conditions, AZD0328, RG3487, and ABT-107 acted as partial agonists, demonstrating 58, 63, 79%, respectively, of the activity of ACh (Sydserff et al., 2009; Malysz et al., 2010; Wallace et al., 2011). TC-5619 was a full agonist (Hauser et al., 2009). The high affinity of EVP-6124 for α7 nAChRs, with an EC_{50} in the range of 0.16–0.39 μM, was similarly observed in other recently described α7 nAChR agonists. In studies reporting peak current, as reported in this study, EC_{50} values ranged between 0.28 and 1.04 μM (Sydserff et al., 2009; Malysz et al., 2010; Wallace et al., 2011). The EC_{50} for TC-5619 of 0.033 μM was reported as net charge, a method that produced a 3 to 6 fold lower value than that produced by peak current analysis, when both analyses were performed in the same studies (Sydserff et al., 2009; Malysz et al., 2010; Wallace et al., 2011). In summary, the EC_{50} values for EVP-6124, as well as the other recently described quinuclidines, suggested that therapeutic concentrations should be expected in the high nanomolar or low micromolar range, if the compounds were to act solely as receptor agonists that required near to full receptor occupancy.

EVP-6124 demonstrated good brain penetration after oral administration, with B:P ratios of approximately 2 between 1 and 4 h and 5 at 8 h. Recently described agonists have varied widely in B:P ratios. RG3487 had the lowest B:P ratio of 0.13, followed by ABT-107 and PHA-543613 (1 and 1.5, respectively) (Wishka et al., 2006; Bitner et al., 2010; Wallace et al., 2011). Of the quinuclidines, PNU-282987 had a high B:P ratio of 5 (Bodnar et al., 2005). The B:P ratio of about 2 for EVP-6124 supported further study of the compound in cognitive tests.

The memory enhancing effects of EVP-6124 *in vivo* were demonstrated in the ORT in a test of short-term memory impairment caused by scopolamine and in a test of natural forgetting, with a 24 h retention interval between the two trials. These data clearly illustrated that EVP-6124 improved memory in a dose-dependent manner, reaching a peak of activity at brain concentrations in the low nanomolar range. Efficacy in cognitive tests at low nanomolar concentrations have similarly been reported for ABT-107 and RG3487 (Bitner et al., 2010; Malysz et al., 2010; Wallace et al., 2011).

Since EVP-6124 (0.3 mg/kg, p.o.) was administered before T1 in the ORT and therefore could have exerted an effect on memory consolidation, as well as on

acquisition, additional studies were performed in which the effect of EVP-6124 on memory consolidation could be investigated. EVP-6124 improved memory consolidation when administered immediately after T1 in an ORT, as was found for RG3487 (Wallace et al., 2011).

In addition, the effect of 0.3 mg/kg of EVP-6124 in the natural forgetting test was blocked by administration of the selective α7 nAChR antagonist MLA (0.3 mg/kg, i.p. or 10 µg, i.c.v.). These studies indicated that the pro-cognitive effect of EVP-6124 was specifically mediated via brain α7 nAChRs. Cognitive improvements by other α7 nAChR agonists were similarly blocked by MLA administered either centrally or peripherally at comparable concentrations (van Kampen et al., 2004; Boess et al., 2007; Pichat et al., 2007; Wallace et al., 2011). Some of the α7 nAChR agonists, including EVP-6124, are also 5-HT_3 receptor antagonists (Boess et al., 2007; Sydserff et al., 2009; Wallace et al., 2011), and may therefore enhance cognition via this latter mechanism (for a review see: Walstab et al., 2010). However, the selective 5-HT_3 receptor antagonist ondansetron (0.1-10 mg/kg, p.o.) did not prevent natural forgetting in an ORT, while the α7 nAChR agonist RG3487 was effective (Wallace et al., 2011). Additionally, the α7 nAChR agonist AZD0328, with a 25 nM K_i at rat 5-HT_3 receptors, was not effective in an ORT in mice lacking α7 nAChRs (Sydserff et al., 2009). Furthermore, the brain concentrations of EVP-6124 and other α7 nAChR agonists required for efficacy in cognitive tests (sub- to low nanomolar) are below the concentrations required for antagonist activity at 5-HT_3 receptors where near to full receptor occupancy would likely be required.

Since treatment with α7 nAChR agonists may benefit AD patients, and they are often treated with an AChEI, we investigated the potential beneficial interaction between AChEIs and EVP-6124. Co-infusion studies of ABT-107 with donepezil demonstrated that the combination of an α7 nAChR agonist and an AChEI was not deleterious to cognition (Bitner et al., 2010). Since the doses of both ABT-107 and donepezil were efficacious alone, a ceiling effect may have precluded the observation of an additive effect of the combination. Accordingly, we tested the combination of previously established sub-efficacious doses of EVP-6124 (0.03 mg/kg, p.o.) and donepezil (0.1 mg/kg, p.o.) and found that the combined treatment completely reversed the scopolamine-induced deficit in the ORT. The results of this combination study further suggested that the pro-cognitive effects of AChEIs, which are dose limited by adverse effects, primarily gastrointestinal (Birks and Flicker, 2006), could be enhanced if AChEIs are combined in low doses with an α7 nAChR agonist in AD patients.

Based on the pharmacokinetic data, estimation of the maximum effective brain concentration in the ORT after a dose of 0.3 mg/kg, p.o. was about 2 nM (total concentration of EVP-6124) and less than 1 nM free drug. Thus, in spite of the high affinity of EVP-6124 for α7 nAChRs, a concentration lower than 3 nM was clearly insufficient to activate the receptor in *in vitro* studies, suggesting an alternative mechanism of action. Moreover, in the animal studies, the EVP-6124 concentration slowly built up and remained stable before and while the behavioral tests were performed. Therefore, these conditions cannot be compared to a brief pulse exposure to EVP-6124 used for the determination of the concentration activation curve *in vitro*.

Since AChEIs inhibit the cleavage of ACh and thereby increase the level of this neurotransmitter, we further examined the interaction of EVP-6124 and ACh at the cellular level in sustained exposure experiments that were aimed at mimicking the conditions of an animal treated with EVP-6124 (Fig. 6A-B). These data clearly illustrated that sustained exposure to a concentration of EVP-6124 below 1 nM potentiated the ACh-evoked current. RG3487 potentiated the ACh-evoked current at 3–10 nM (Wallace et al., 2011). Increasing the EVP-6124 concentration to 3 nM or above, caused a marked reduction of the ACh-evoked current that was attributable to receptor desensitization. Under similar experimental conditions of paired application of an α7 nAChR agonist and ACh, α7 nAChR desensitization was observed in the low nanomolar range with ABT-107 and RG3487 at 10 and 30 nM, respectively (Malysz et al., 2010; Wallace et al., 2011). The pro-cognitive effects after EVP-6124, ABT-107, and RG3487 administration appear to have occurred at brain concentrations below those that caused desensitization. Reducing the frequency of stimulation diminished the desensitization caused by successive agonist exposures and revealed a larger degree of potentiation (Fig. 6C-D). These data illustrated that the presence of a low concentration of EVP-6124, in the sub-nanomolar range increased the ACh-evoked current by a factor of two or more.

Although different mechanisms can be postulated to account for the observed potentiation, the simplest and most probable model considers the co-agonistic behavior of EVP-6124 and ACh at α7 nAChRs. In light of our findings in oocytes treated with EVP-6124 and ACh in sustained and intermittent exposure experiments, we were directed to a model of co-agonist activity described for tubocurarine and ACh at α3β4 nAChRs (Cachelin and Rust, 1994). Following this initial description, additional evidence for co-agonist activities was reported for other nAChRs subtypes (Zwart et al., 2000; Smulders et al., 2005; Papke et al. 2011; Wallace et al. 2011). In this model, a single receptor displays at least two

pockets where the ligand can bind. Similarly, two molecules of ACh must bind to the α7 nAChR to activate it. Exposure to a low concentration of a high affinity ligand, such as EVP-6124, will increase the probability that a single molecule of this ligand occupies the receptor. As occupancy of the receptor by a single molecule is assumed to be insufficient to activate the receptor, exposure to such a low concentration of ligand is not expected to cause channel opening. Brief and intermittent exposure to another ligand with lower affinity, such as ACh, will then trigger channel opening and an inward current. These steps, which correspond to the model formulated by Cachelin and Rust (1994), are summarized in Fig. 7.

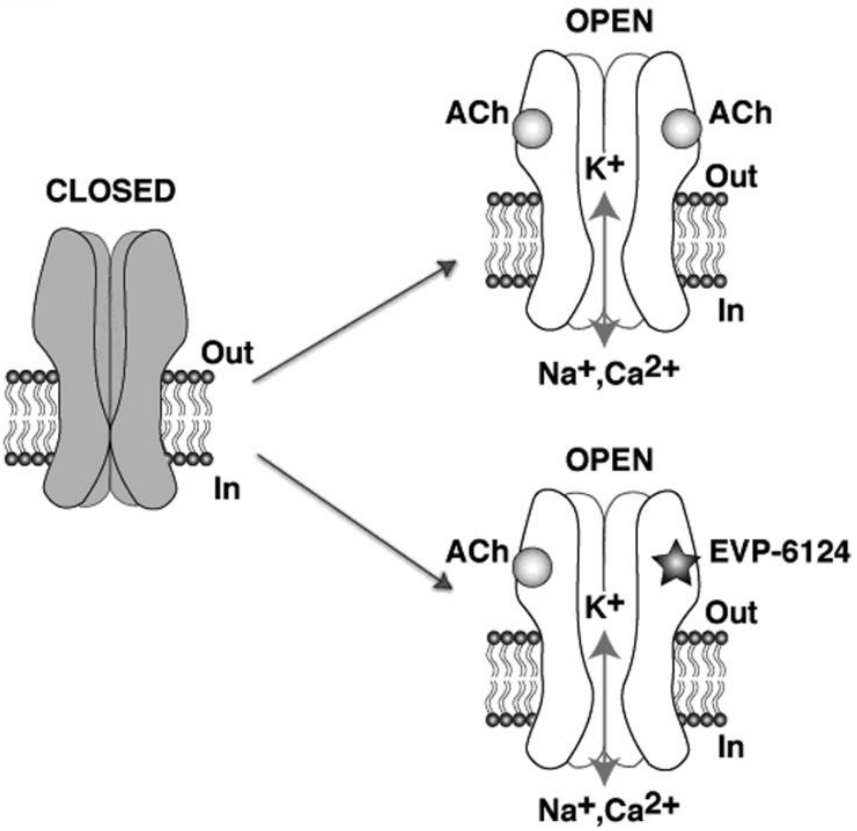


Figure 7. Model of EVP-6124 and ACh binding. In the absence of ligand the receptor is closed (left panel). ACh applications allowing two (or more) molecules to bind and activate the receptor (upper right panel). Exposure to a low concentration of EVP-6124 causes binding of one EVP-6124 molecule on the receptor that is insufficient to activate the receptor. Brisk activation of the receptor will, however, occur upon exposure to ACh and a single molecule might be sufficient to trigger the opening of the receptor (lower right panel).

Altogether, these data provide evidence that the novel synthetic compound, EVP-6124, acted as a potent partial and selective agonist at α7 nAChRs. Exposure of EVP-6124 to α7 nAChRs at a sub- to low nanomolar range, which corresponded to the active doses in behavioral tests, caused a potentiation of the ACh-evoked current that can account for the pro-cognitive effects observed in animals. These data suggested that a novel mechanism of action at low agonist concentrations of EVP-6124 was responsible for its pro-cognitive effects, a notion that needs to be investigated further in controlled experiments.

The finding of a novel mechanism of action of a partial agonist acting at a concentration in the sub-nanomolar range through a co-agonism mechanism may lead to a more desirable side-effect profile than more classical approaches which dictate that the drug be dosed to full agonist concentrations. Activation of α7 nAChRs by exposure to a low agonist concentration, of a drug such as EVP-6124 utilizing this co-agonist mechanism, is expected to increase the drug safety margin, to minimize undesired interactions with other receptors, and to open new and promising therapeutic avenues in combination with classical AChEIs at lower than typically prescribed doses.

Acknowledgements
We thank E. Neveu for technical assistance in experiments and E. Bollen for technical assistance with the stereotaxic surgeries.

References

Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., Rogers, S. W. (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiological Reviews 89*(1), 73-120.

Arendash, G. W., Sengstock, G. J., Sanberg, P. R., Kem, W. R. (1995). Improved learning and memory in aged rats with chronic administration of the nicotinic receptor agonist GTS-21. *Brain Research. 674*(2), 252-259.

Beck, K. D., and Luine, V. N. (2002). Sex differences in behavioral and neurochemical profiles after chronic stress: role of housing conditions. *Physiology & Behavior 75*(5), 661-673.

Birks, J., and Flicker, L. (2006). Donepezil for mild cognitive impairment. Cochrane Database Syst. Rev. Issue 3. Art. No.: CD006104. DOI: 10.1002/14651858.CD006104.

Bitner, R. S., Bunnelle, W. H., Anderson, D. J., Briggs, C. A., Buccafusco, J., Curzon, P., et al. (2007). Broad-spectrum efficacy across cognitive domains by α7 nicotinic acetylcholine receptor agonism correlates with activation of ERK1/2 and CREB phosphorylation pathways. *Journal of Neuroscience, 27*(39), 10578-10587.

Bitner, R. S., Bunnelle, W. H., Decker, M. W., Drescher, K. U., Kohlhaas, K. L., Markosyan, S., et al. (2010). In vivo pharmacological characterization of a novel selective α7 neuronal nicotinic acetylcholine receptor agonist ABT-107: preclinical considerations in Alzheimer's disease. *Journal of Pharmacology and Experimental Therapeutics, 334*(3), 875-886.

Biton, B., Bergis, O. E., Galli, F., Nedelec, A., Lochead, A. W., Jegham, S., et al. (2007). SSR180711, a novel selective α7 nicotinic receptor partial agonist: (I) binding and functional profile. *Neuropsychopharmacology, 32*, 1-16.

Bodnar, A. L., Cortes-Burgos, L. A., Cook, K. K., Dinh, D. M., Groppi, V. E., Hajos, M., et al. (2005). Discovery and structure–activity relationship of quinuclidine benzamides as agonists of α7 nicotinic acetylcholine receptors. *Journal of Medicinal Chemistry, 48*(4), 905-908.

Boess, F. G., De Vry, J., Erb, C., Flessner, T., Hendrix, M., Luithle, J., et al. (2007). The novel α7 nicotinic acetylcholine receptor agonist *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide improves working and recognition memory in rodents. *Journal of Pharmacology and Experimental Therapeutics, 321*(2), 716-725.

Briggs, C. A., Anderson, D. J., Brioni, J. D., Buccafusco, J. J., Buckley, M. J., Campbell, J. E., et al. (1997). Functional characterization of the novel neuronal nicotinic acetylcholine receptor ligand GTS-21 in vitro and in vivo. *Pharmacology Biochemistry and Behavior, 57*(1-2), 231-241.

Briggs, C. A., Grønlien, J. H., Curzon, P., Timmermann, D. B., Ween H., Thorin-Hagene, K., et al. (2009). Role of channel activation in cognitive enhancement mediated by α7 nicotinic acetylcholine receptors. *British Journal of Pharmacology, 158*(6), 1486-1494.

Cachelin, A. B., and Rust, G. (1994). Unusual pharmacology of (+)-tubocurarine with rat neuronal nicotinic acetylcholine receptors containing beta 4 subunits. *Molecular Pharmacology, 46*(6), 1168-1174.

Dani, J. A., and Bertrand, D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews Pharmacology and Toxicology, 47*, 699-729.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behavioural Brain Research, 31*(1), 47-59.

Feuerbach, D., Lingenhoehl, K., Olpe, H. R., Vassout, A., Gentsch, C., Chaperon, F., et al. (2009). The selective nicotinic acetylcholine receptor α7 agonist JN403 is active in animal models of cognition, sensory gating, epilepsy and pain. *Neuropharmacology, 56*(1), 254-263.

Hauser, T. A., Kucinski, A., Jordan, K. G., Gatto, G. J., Wersinger, S. R., Hesse, R. A., et al. (2009). TC-5619: an alpha7 neuronal nicotinic receptor-selective agonist that demonstrates efficacy in animal models of the positive and negative symptoms and cognitive dysfunction of schizophrenia. *Biochemical Pharmacology, 78*(7), 803-812.

Hogg, R. C., Bandelier, F., Benoit, A., Dosch, R., Bertrand, D. (2008). An automated system for intracellular and intranuclear injection. *Journal of Neuroscience Methods, 169*(1)**,** 65-75.

Hunter, B. E., de Fiebre, C. M., Papke, R. L., Kem, W. R., Meyer, E. M. (1994). A novel nicotinic agonist facilitates induction of long-term potentiation in the rat hippocampus. *Neuroscience Letters, 168*(1-2), 130-134.

Kem, W.R. (2000). The brain α7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). *Behavioural Brain Research, 113*(1-2), 169-181.

Kitagawa, H., Takenouchi, T., Azuma, R., Wesnes, K. A., Kramer, W. G., Clody, D. E., et al. (2003). Safety, pharmacokinetics, and effects on cognitive function of multiple doses of GTS-21 in healthy, male volunteers. *Neuropsychopharmacology, 28*, 542-551.

Lagostena, L., Trocme-Thibierge, C., Morain, P., Cherubini, E. (2008). The partial α7 nicotine acetylcholine receptor agonist S 24795 enhances long-term potentiation at CA3-CA1 synapses in the adult mouse hippocampus. *Neuropharmacology, 54*(4), 676-685.

Levin, E. D., Bettegowda, C., Blosser, J., Gordon, J. (1999). AR-R17779, an [alpha]7 nicotinic agonist, improves learning and memory in rats. *Behavioural Pharmacology, 10*(6-7), 675-680.

Levin, E. D., McClernon, F. J., Rezvani, A. H. (2006). Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology, 184*(3-4), 523-539.

Malysz, J., Anderson, D. J., Grønlien, J. H., Ji, J., Bunnelle, W. H., Håkerud, M., et al. (2010). In vitro pharmacological characterization of a novel selective α7 neuronal nicotinic acetylcholine receptor agonist ABT-107. *Journal of Pharmacology and Experimental Therapeutics, 334*(3), 863-874.

Meyer, E. M., de Fiebre, C. M., Hunter, B. E., Simpkins, C. E., Frauworth, N., de Fiebre, N. E. C. (1994). Effects of anabaseine-related analogs on rat brain nicotinic receptor binding and on avoidance behaviors. *Drug Development Research, 31*(2), 127-134.

Olincy, A., Harris, J. G., Johnson, L. L., Pender, V., Kongs, S., Allensworth, D., et al. (2006). Proof-of-concept trial of an α7 nicotinic agonist in schizophrenia. *Archives of General Psychiatry, 63*(6), 630-638.

Papke, R. L., Trocmé-Thibierge, C., Guendisch, D., Al Rubaiy, S. A. A., Bloom, S. A. (2011). Electrophysiological perspectives on the therapeutic use of nicotinic acetylcholine receptor partial agonists. *Journal of Pharmacology and Experimental Therapeutics, 337*(2), 367-379.

Paxinos, G., and Watson, C. (1996). The Rat Brain in Stereotaxic Coordinates, fourth ed. *Academic Press,* London.

Pichat, P., Bergis, O. E., Terranova, J. P., Urani, A., Duarte, C., Santucci, V., et al. (2007). SSR180711, a novel selective α7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. *Neuropsychopharmacology, 32,* 17-34.

Ponganis, K. V., and Stanski, D. R. (1985). Factors affecting the measurement of lidocaine protein binding by equilibrium dialysis in human serum. *Journal of Pharmaceutical Sciences, 74*(1), 57-60.

Prickaerts, J., Steinbusch, H. W. M., Smits, J. F. M., de Vente, J. (1997). Possible role of nitric oxide-cyclic GMP pathway in object recognition memory: effects of 7-nitroindazole and zaprinast. *European Journal of Pharmacology, 337*(2-3), 125-136.

Roncarati, R., Scali, C., Comery, T. A., Grauer, S. M., Aschmi, S., Bothmann, H., et al. (2009). Procognitive and neuroprotective activity of a novel α7 nicotinic acetylcholine receptor agonist for treatment of neurodegenerative and cognitive disorders. *Journal of Pharmacology and Experimental Therapeutics, 329*(2), 459-468.

Rutten, K., Prickaerts, J., Hendrix, M., van der Staay, F. J., Şik, A., Blokland, A. (2007). Time-dependent involvement of cAMP and cGMP in consolidation of object memory: studies using selective phosphodiesterase type 2, 4 and 5 inhibitors. *European Journal of Pharmacology, 558*(1-3), 107-112.

Smulders, C. J. G. M., Zwart, R., Bermudez, I., van Kleef, R. G. D. M., Groot-Kormelink, P. J., Vijverberg, H. P. M. (2005). Cholinergic drugs potentiate human nicotinic α4β2 acetylcholine receptors by a competitive mechanism. *European Journal of Pharmacology, 509*(2-3), 97-108.

Sydserff, S., Sutton, E. J., Song, D., Quirk, M. C., Maciag, C., Li, C., et al. (2009). Selective α7 nicotinic receptor activation by AZD0328 enhances cortical dopamine release and improves learning and attentional processes. *Biochemical Pharmacology, 78*(7), 880-888.

van Kampen, M., Selbach, K., Schneider, R., Schiegel, E., Boess, F., Schreiber, R. (2004). AR-R 17779 improves social recognition in rats by activation of nicotinic α_7 receptors. *Psychopharmacology, 172*(4), 375-383.

Wallace, T. L., Callahan, P. M., Tehim, A., Bertrand, D., Tombaugh, G., Wang, S., et al. (2011). RG3487, a novel nicotinic α7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. *Journal of Pharmacology and Experimental Therapeutics, 336*(1), 242-253.

Walstab, J., Rappold, G., Niesler, B. (2010). 5-HT_3 receptors: role in disease and target of drugs. *Pharmacology & Therapeutics, 128*(1), 146-169.

Wishka, D. G., Walker, D. P., Yates, K. M., Reitz, S. C., Jia, S., Myers, J. K., et al. (2006). Discovery of *N*-[(3*R*)-1-azabicyclo[2.2.2]oct-3-yl]furo[2,3-*c*]pyridine-5-carboxamide, an agonist of the α7 nicotinic acetylcholine receptor, for the potential treatment of cognitive deficits in schizophrenia: synthesis and structure–activity relationship. *Journal of Medicinal Chemistry, 49*(14), 4425-4436.

Zwart, R., van Kleef, R. G. D. M., Gotti, C., Smulders, C. J. G. M., Vijverberg, H. P. M. (2000). Competitive potentiation of acetylcholine effects on neuronal nicotinic receptors by acetylcholinesterase-inhibiting drugs. *Journal of Neurochemistry, 75*(6), 2492-2500.

Chapter 6

Continuous infusion of the α7 nicotinic receptor partial gonist EVP-6124 produces no signs of tolerance at memory enhancing doses in rats: A pharmacokinetic and behavioral study

Nick P. van Goethem, Jos Prickaerts, Devin Welty,
Dorothy G. Flood and Gerhard Koenig
Behavioural Pharmacology (2015), 26(4), 403-406

Abstract

Objectives. We investigated the effect of acute and six-day continuous minipump administration of the α7 nAChR agonist EVP-6124 on memory performance in rats.

Methods. Memory performance in a natural forgetting test (i.e. a 24-h retention interval) was measured in the object recognition task (ORT). First, a dose-response study with a single-oral dose of EVP-6124 at 0.3 or 1 mg/kg was performed. Second, we investigated whether a procognitive effect was still observed after six days of continuous EVP-6124 treatment. The targeted plasma steady state concentrations (C_{ss}) were 0.6 and 2 ng/ml, which were similar to the efficacious plasma concentrations after an acute oral dose.

Results. The optimal acute dose of EVP-6124 for enhancing memory was 0.3 mg/kg. 1 mg/kg showed an intermediate procognitive effect. The plasma concentration necessary for optimally enhancing memory after a single-oral dose of EVP-6124 is approximately 0.3 ng/ml at 1-4 h post-dosing. Six days of continuous treatment with EVP-6124 resulted in measured plasma C_{ss} values of 0.48 and 1.93 ng/ml on day 6. Both measured plasma C_{ss} values enhanced memory.

Conclusions. This indicates that at EVP-6124 plasma concentrations that optimally enhance memory in the ORT, tolerance did not develop after six days of continuous treatment.

Introduction

Administration of nicotine has been shown to improve cognitive functioning, mainly related to learning, memory and attention, in both animal (Levin and Simon, 1998) and human studies (Newhouse et al., 1997; Newhouse et al., 2004), through interaction with neuronal nicotinic acetylcholine receptors (nAChRs) (e.g. see Toyohara and Hashimoto, 2010). nAChRs are members of the super family of ligand-gated ion channels, and are distributed throughout the central nervous system (CNS). Each nAChR consists of five subunits, and in the CNS these subunits can either be of the α or the β subtype. Respectively, eight and three different subunits of these subtypes have been identified at present (α2 – α10 and β2 – β4). This allows for multiple combinations of these α and β subunits to generate many different nAChR subtypes, both homopentameric (solely α subunits) and heteropentameric (combinations of α and β subunits) nAChRs. These different receptor compositions have their own specific anatomical distributions and pharmacologies (Dani, 2001).

In the CNS, two major subtypes of nAChRs have been described, the homopentameric α7 nAChR and the heteropentameric α4β2 nAChR. The former has a lower affinity for nicotine and their endogenous neurotransmitter acetylcholine (ACh) when compared to the latter (Dani and Bertrand, 2007; Albuquerque et al., 2009). The α7 nAChRs are highly expressed in the hippocampus, a brain region implicated in several types of memory. In addition, α7 nAChRs are directly involved in hippocampal long-term potentiation and theta oscillations, the putative cellular mechanisms underlying learning and memory (Mansvelder and McGehee, 2000; Siok et al., 2006). Because of these features, the α7 nAChR is a promising target for the improvement of cognition in disorders in which cognition is compromised. Different α7 nAChR agonists have been investigated for their potential to improve memory and attention deficits encountered in for example Alzheimer's disease (AD) and schizophrenia (Toyohara and Hashimoto, 2010; Wallace and Porter, 2011). At present, many such agonists have been developed and tested both in animals (e.g. Prickaerts et al., 2012) and in humans (e.g. Othman et al., 2011; Preskorn et al., 2014).

In our previous *in vitro* and *in vivo* studies, EVP-6124 ((*R*)-7-chloro-*N*-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide) was shown to have selectivity for α7 nAChRs, where it had partial agonistic effects, with maximum current amplitude of 42 ± 3% of ACh (Prickaerts et al., 2012). EVP-6124 was approximately 300-fold more potent than the natural ligand ACh (K_i=3 μM), as measured in binding assays using [^{3}H]-methyllycaconitine. Furthermore, EVP-6124 did not activate or inhibit α4β2 nAChRs, as indicated by the absence of displacement of [^{3}H]-

cytisine from α4β2 nAChRs by 10 μM EVP-6124. In co-application *in vitro* experiments of EVP-6124 with ACh, sustained exposure to EVP-6124 in oocytes caused desensitization at concentrations greater than 3 nM, while lower concentrations (0.3–1 nM [0.1-0.3 ng/ml]) caused an increase in the ACh-evoked response. *In vivo*, behavioral studies showed that acute EVP-6124 treatment improved memory performance of rats in the object recognition task (ORT). Furthermore, it was found that an acute sub-efficacious dose of EVP-6124 co-administered with a sub-efficacious dose of the acetylcholinesterase inhibitor donepezil fully restored memory of rats as well. Evidence from both of these *in vitro* and *in vivo* studies was interpreted as a co-agonistic activity of EVP-6124 with ACh on α7 nAChRs. This suggested that EVP-6124 improves memory performance of rats by potentiating the ACh response of α7 nAChRs (Prickaerts et al., 2012). Recently, EVP-6124 was found to improve performance of patients with schizophrenia, who were already on antipsychotic medication, on several cognitive tests related to memory and learning or executive functioning. Based on the findings of this proof-of-concept randomized trial, larger scale studies were initiated to further test the pro-cognitive effects of EVP-6124 in patients with schizophrenia (Preskorn et al., 2014).

α7 nAChRs have a high permeability to Ca^{2+} and show rapid desensitization following exposure to agonists (Picciotto et al., 2000). Desensitization of these receptors has raised the question whether the continuous presence of α7 nAChR agonists would retain the efficacy observed in acute dosing studies in animal models of cognition. Desensitization of these receptors could imply tolerance would develop on a behavioral level, i.e. the memory enhancing effects observed in memory tasks with acute administration might not be retained after chronic exposure to α7 nAChR agonists. In an earlier study, Bitner et al., (2010) showed that 7 days of continuous infusion with the selective α7 nAChR agonist ABT-107 significantly improved rat performance in a social recognition model. The tested plasma steady state concentration (C_{ss}) in this study was 0.2 ng/ml. These results suggest a lack of tolerance development to the cognition enhancing effect of this compound within this C_{ss} range over 7 days in rats.

In this work, we investigated whether the procognitive acute effect of a single-dose administration of EVP-6124, was maintained after six days of minipump administration to C_{ss} in rats. In other words, we investigated on a behavioral level whether tolerance to the cognition enhancing effects of EVP-6124 after six days of continuous treatment would develop. As in the previous study (Prickaerts et al., 2012), a natural forgetting test (i.e. a 24-h retention interval)

was utilized in the ORT. Both the acute and the six-day continuous exposure to different doses of EVP-6124 were assessed. From this previous study it is known that the plasma concentration necessary for enhancing memory after a single-oral dose of EVP-6124 is approximately 0.3 ng/ml at 1-4 h post-dosing in rats. In the single-dose administration study, doses of 0.3 and 1.0 mg/kg were chosen. To replicate the aforementioned findings, and to investigate whether tolerance to EVP-6124 develops, total plasma C_{ss} values of 0.6 and 2.0 ng/ml were targeted in the (sub)chronic study. To ascertain that the intended plasma C_{ss} values were indeed tested, a pilot pharmacokinetic (PK) study was conducted before the actual (sub)chronic study was performed.

Materials and methods

Materials/reagents

EVP-6124·HCl·H_2O (lot number 022376-C-02) was synthesized by Ricerca Biosciences (Concord, OH). Vehicle was sterile physiological saline (B. Braun Melsungen AG, Melsungen, Germany).

Animals

All experimental procedures were approved by the local ethics committee of Maastricht University for animal experiments and met governmental guidelines of The Netherlands. For the pilot PK study, twelve 4-month-old male Wistar rats were used. For the ORT studies using single-dose oral (p.o.) and continuous minipump (Alzet, model 2001, Charles River, Germany) administration, forty-eight 3-month-old male Wistar rats were used. For all studies, the rats were obtained from Charles River Laboratories International, Inc. (Sulzfeld, Germany). Rats weighed on average 398-436 grams at the beginning of the studies, and were housed individually (van Goethem et al., 2012) in standard Tecniplast IVC system greenline cages on sawdust bedding. The animals were on a reversed 12/12-h light/dark cycle (lights on from 19:00 to 7:00 h); and food and water were given ad libitum. The rats were housed and tested in the same room. A radio, playing softly, provided background noise to mask noises in the room. All testing was performed between 9:00 and 18:00 h under low illumination (20 lux).

Treatment

Pilot PK study (four-day continuous EVP-6124 treatment by means of minipump administration)

EVP-6124·HCl·H_2O was dissolved in saline and was adjusted to reflect the weight of the EVP-6124 free base. The correction factor for difference in molecular

weight of anhydrous EVP-6124 free-base versus EVP-6124·HCl·H_2O is: 375.3 (HCl·H_2O) / 320.8 (anhydrous free base) = 1.17. In both experimental groups (0.6 and 2.0 ng/ml), six rats were used. Solutions were prepared to target total C_{ss} values of 0.6 and 2.0 ng/ml (unbound C_{ss} values of approximately 0.07 and 0.2 ng/ml) using the equation below, and an EVP-6124 clearance value in rat obtained in a prior PK study (8300 ml/h/kg). The calculations for compound preparation are given in Table 1.

$$\text{Rate of infusion} = C_{ss} \bullet \text{Clearance}$$

Table 1. Calculations for Compound Preparation (Pilot PK study)

Target plasma C_{ss} (ng/ml)	Mean weight of rats (kg)	EVP-6124 infusion rate (µg/h)	Corrected for salt weight (• 1.170)	Concentration per pump (2 pumps: µg/µl)
0.6	0.436	2.171	2.540	1.27
2.0	0.434	7.204	8.428	4.21

Alzet osmotic minipump model 2001, reservoir volume: 200 µL, delivery rate: 1 µL/h. Two pumps per rat were placed. n = 6/group.

Single-oral dose EVP-6124 treatment

EVP-6124·HCl·H_2O was dissolved in saline to produce an injection volume of 2 ml/kg. The dose was calculated from the weight of EVP-6124·HCl·H_2O, in order to replicate the procedures of the previous study (Prickaerts et al., 2012). Samples were taken from the dosing solutions, pre- and post-dosing, for analysis. Single doses of 0.3 and 1.0 mg/kg of EVP-6124 or vehicle were administered p.o., 30 min before the first (learning) trial (T1) of the ORT. In all three experimental conditions (vehicle/control, 0.3 and 1.0 mg/kg) sixteen rats were used. The animals were randomly assigned to each of the three experimental conditions.

Six-day continuous EVP-6124 treatment by means of minipump administration

EVP-6124·HCl·H_2O was prepared as described above for the pilot PK study, using the correction factor of 1.17. In all three experimental conditions

(vehicle/control, 0.6 and 2.0 ng/ml) sixteen rats were used. The calculations for compound preparation are given in Table 2.

Table 2. Calculations for Compound Preparation (Six-Day Continuous EVP-6124 Treatment with ORT)

Target plasma C_{ss} (ng/ml)	Mean weight of rats (kg)	EVP-6124 infusion rate (µg/h)	Corrected for salt weight (• 1.170)	Concentration per pump (2 pumps: µg/µL)
Vehicle	0.398	0.00	0.00	0.00
0.6	0.401	1.997	2.336	1.17
2.0	0.403	6.690	7.827	3.91

Alzet osmotic minipump model 2001, reservoir volume: 200 µL, delivery rate: 1 µL/h. Two pumps per rat were placed. n = 16/group.

Object recognition memory task

Apparatus

The ORT was performed as described elsewhere (Ennaceur and Delacour, 1988; Prickaerts et al., 2012). The apparatus consisted of a circular arena, 83 cm in diameter. Half of the 40 cm high wall was made of gray polyvinyl chloride, the other half of transparent polyvinyl chloride. Fluorescent red tubes and a light bulb provided a constant illumination of about 20 lux on the floor of the apparatus.

Objects

Two objects were placed in a symmetrical position about 10 cm away from the gray wall. Four objects were used: 1) a cone consisting of a gray polyvinyl chloride base (maximal diameter 18 cm) with a collar on top made of brass (total height 16 cm), 2) a standard 1 L brown transparent glass bottle (diameter 10 cm, height 22 cm) filled with water, 3) a metal block (10.0 x 5.0 x 7.5 cm) with two holes (diameter 1.9 cm), and 4) an aluminum block with a tapering top (13.0 x 8.0 x 8.0 cm). Each object was available in triplicate so that a rat did not explore an object it had already explored in T1 during the second trial (T2). This way, potential traces that could attract the rat towards the familiar object were excluded. A rat could not displace the objects.

Object recognition memory task

A testing session comprised two trials (T1 and T2), each with durations of 3 min. During T1 (the learning trial) the apparatus contained two identical objects (samples). A rat was always placed in the apparatus facing the wall at the middle of the front (transparent) segment. After T1 the rat was put back in its home cage for a 24-h inter-trial interval. Subsequently, the rat was put back in the apparatus for T2 (the test trial), but now with a familiar object from T1 (the sample) and a new object. The times spent in exploring each object during T1 and T2 were recorded manually with a personal computer. The experimenter was blind to the treatment group to which rats were assigned.

Exploration was defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with the nose. Sitting or leaning on an object was not considered as exploratory behavior. In order to avoid the presence of olfactory cues, the objects were thoroughly cleaned after each trial. All combinations and locations of objects were used in a balanced manner to reduce possible biases due to preferences for particular locations or objects.

Animal handling

Prior to compound testing studies, the animals were handled daily, adapted to the procedures (also p.o. injections, 2 ml/kg, saline), and allowed to explore the apparatus and objects twice for 3 min each day for 2 days.

First, a study with single-oral doses of EVP-6124 was performed, followed by the six-day continuous EVP-6124 treatment by means of minipump administration. Eight days after the completion of the single-dose study, minipumps were implanted. This allowed enough time for the compound to be washed out of the rats ($t_{1/2}$ of EVP-6124 in rats ~1-2 h). After five days of continuous EVP-6124 treatment via minipump administration, T1 of a 24-h interval ORT was performed. On day 6, T2 was performed, and blood and solution samples from the minipumps were taken for analysis.

Statistical analysis

The ORT provides measures for exploration time and discrimination (Prickaerts et al., 1997). The measures were the times spent by rats in exploring each object during T1 and T2. The time spent in exploring the two identical samples in T1 were represented by ‘a1’ and ‘a2’, respectively. The time spent in exploring the sample and the new object in T2 were represented by ‘a3’ and ‘b’, respectively. From these exploration times, the total exploration times for T1 and T2 were

calculated: e1 = a1 + a2 and e2 = a3 + b. The d2 index is a relative measure of discrimination corrected for exploratory activity, where d2 = (b – a3) / e2. The d2 index can range from -1 to 1, with -1 or 1 indicating complete preference for the familiar or novel object, respectively, and 0 signifying no preference for either object.

One-sample *t*-tests were performed in order to assess whether the d2 index for each treatment group separately differed significantly from zero, i.e. chance level. In addition, treatment groups were also compared using one-way analysis of variance (ANOVA). When the overall ANOVA was significant, a post-hoc analysis with Bonferroni *t*-tests (all pairwise comparisons) was performed. An α level of 0.05 was considered significant. Data are expressed as means and standard error of the mean (SEM).

Minipump preparation and implantation

Body weights of the rats were recorded and used to determine the concentration of the solution in the minipumps. Osmotic minipumps (Alzet, Charles River, Germany) were filled according to the manufacturer's instructions. The osmotic minipumps were able to deliver drug solution for 200 h, or approximately 8 days.

The rats underwent one surgical procedure in which the minipumps were placed subcutaneously in the flanks of the rats (one minipump on each side). To establish more comfortable continuous drug administration, it was decided to place two small pumps instead of one large pump in the animals. The rats received a subcutaneous injection of the analgesic Temgesic (buprenorphine, 0.05 mg/kg, s.c.) 30 min before the surgical procedure. In order to fully anesthetize the rats, they were placed in an induction unit which was filled with a mixture of air and 5% isoflurane. Following induction, anesthesia was maintained with 2% isoflurane from a mask placed over the rat's snout. Before the procedure started, the hind paw withdrawal reflex was assessed and was absent. The eyes were treated with Vaseline ointment in order to prevent dehydration. Furthermore, the rat was constantly checked in order to determine whether the breathing was deep and regular. A small incision was made on both flanks of the rat, after which the minipumps (length: 3.0 cm and diameter: 0.7 cm) were subcutaneously inserted. After placing the minipumps, the small wounds were immediately closed with sutures. Upon recovery from anesthesia, rats were returned to their home cages and observed daily for the entire experiment.

Blood/plasma collection procedure

Rat blood was collected in order to determine the plasma concentrations of EVP-6124 on days 2, 3, and 4 (where day 1 was the minipump implantation surgery) in the pilot PK study and on day 6 after minipump implantation in the behavioral ORT study with six-day continuous minipump administration. This allowed adequate time for the minipumps to equilibrate in the body and for more than 4-5 half-lives of EVP-6124 ($t_{1/2}$ ~ 1-2 h in rat) to reach plasma C_{ss}. About 100 µl of blood was collected via a saphenous vena puncture in blood collection tubes with K_2 EDTA anticoagulant (Microcuvette CB300, Sarstedt, Germany), kept on ice and then centrifuged within 15 min of collection. Blood was centrifuged for 10 min at 4°C and 4000 rpm after which approximately 50 µl of plasma was collected per sample. Dosing solutions were removed from the minipumps as well. Samples were stored frozen at -80°C until analysis.

Analysis of EVP-6124 concentrations in plasma and dosing solutions

Following addition of an internal standard (EVP-6124-D_6, PepTech, Burlington, MA) and methanol, the plasma samples, dosing solutions, and calibration standards were prepared by solid phase extraction (Oasis HLB 96-Well Plate, Waters, Milford, MA). Samples were washed sequentially with 1% ammonium hydroxide and 10% acetonitrile, eluted from the solid phase with 1:100 (v:v) formic acid:acetonitrile, dried, and reconstituted in 80% acetonitrile for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LC system consisted of an Agilent 1200SL binary pump and Thermo HTC PAL autosampler (Agilent Technologies, Santa Clara, CA). The system used a Zorbax 300-SCX 5 µm column (2.1 x 50 mm, Agilent) maintained at 50°C. The mobile phase was 12:88 (v:v) 25 mM ammonium acetate in water:acetonitrile. The mass spectrometer was an API5500 (Applied Biosystems, Foster City, CA) using electrospray ionization (positive-ion mode) and multiple-reaction monitoring scan mode and was optimized for detection of EVP-6124 with a mass transition of 321.1>110.1 and of EVP-6124-D_6 of 327.1>113.3 m/z.

Results

Pilot EVP-6124 PK study

Rats were continuously treated for four days with EVP-6124 by means of minipump administration and blood was taken on days 2, 3 and 4 after minipump placement. The means (± SEM) of the measured concentrations of EVP-6124 in rat plasma are summarized in Table 3 and were similar to the targeted C_{ss} values. No modifications were made to the equations for

determining targeted plasma C_{ss} for the ORT minipump study, other than the change in the average body weights of the rats.

Table 3. EVP-6124 Concentrations (ng/ml) in Rat Plasma (Pilot PK Study)

Targeted plasma C_{ss} of EVP-6124 (ng/ml)	Actual mean (± SEM) concentration of EVP-6124 (ng/ml)		
	Day 2	Day 3	Day 4
0.6	0.627 (0.047)	0.629 (0.050)	0.764 (0.080)
2.0	2.02 (0.17)	2.18 (0.14)	2.23 (0.14)

Data reflect the concentration of the free base. The results of the analysis of the concentrations of the solutions prior to filling the minipumps and in the minipumps sampled on Day 4 were as targeted (data not shown). n = 6 rats per group.

Single-oral dose EVP-6124 study

The results of the single-oral dose study of EVP-6124, administered 30 min before T1, are summarized in Table 4. There were no differences between the treatment groups in the level of exploration in T1 (e1: $F_{2,45}$=0.65, n.s.) or T2 (e2: $F_{2,45}$=0.55, n.s.).

One-sample *t*-tests showed that the d2 indices of the 0.3 and 1.0 mg/kg EVP-6124 groups, but not of the vehicle group, significantly differed from zero (Table 5 and Fig. 1). When between groups comparisons were performed by ANOVA, differences were found for the d2 indices ($F_{2,45}$=6.94, P<0.01). Post-hoc analysis revealed that the d2 index was significantly higher in the 0.3 mg/kg EVP-6124 group when compared to the vehicle group (Fig. 1). Therefore it was concluded that 0.3 mg/kg of EVP-6124 was the better single-oral dose for the prevention of natural forgetting, since this group significantly differed both from zero and the vehicle group while the 1.0 mg/kg dose only differed significantly from zero.

Table 4. Mean (± SEM) Exploration Times and d2 Index in the Natural Forgetting ORT after Single-Oral Doses of EVP-6124

EVP-6124 (mg/kg, p.o.)	e1 (sec)	e2 (sec)	d2 index
Vehicle	26.73 (1.47)	35.56 (2.47)	-0.06 (0.07)
0.3	24.76 (1.27)	32.70 (1.54)	0.26 (0.06)$^{###}$
1.0	26.92 (1.67)	34.41 (1.70)	0.12 (0.04)$^{#}$

Dose was based on the weight of the salt. The EVP-6124 concentrations in dose formulations taken pre- and post-dosing for acute oral dosing were as targeted (data not shown). The d2 index differed from zero by one-sample *t*-tests: $^{#}P<0.05$; $^{###}P<0.001$.

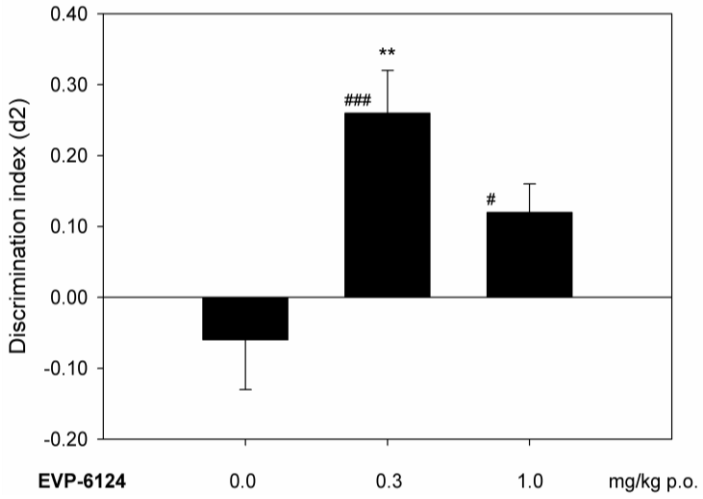


Figure 1. Mean d2 index in the natural forgetting ORT after single-oral doses of EVP-6124. When compared with the vehicle group (0.0, EVP-6124), 0.3 mg/kg (p.o.) of EVP-6124 prevented natural forgetting. A significant post-hoc difference from the vehicle group, following one-way ANOVA: ***P*<0.01. A significant difference from zero in one-sample *t*-tests: #*P*<0.05, ###*P*<0.001. n = 16 animals per group. Error bars represent SEM.

Six-day continuous EVP-6124 treatment by means of minipump administration

The results of the six-day continuous EVP-6124 treatment by means of minipump administration are summarized in Table 5. There were no differences between the treatment groups in the level of exploration in T1 (e1: $F_{2,45}$=2.39, n.s.) or in T2 (e2: $F_{2,45}$=0.25, n.s.).

One-sample *t*-tests showed significant differences from zero for the d2 indices of the two experimental EVP-6124 groups (0.6 and 2.0 ng/ml), indicating recognition of the familiar object and prevention of natural forgetting (Table 5 and Fig. 2). When between groups comparisons were performed, there were differences for the d2 indices ($F_{2,45}$=11.29, P<0.001). Post-hoc analysis revealed that the d2 indices were significantly higher in the 0.6 and 2.0 ng/ml EVP-6124 groups when compared to the vehicle group (Fig. 2).

The means (± SEM) of the measured concentrations of EVP-6124 in rat plasma on day 6 of this study were again as expected and were: 0.0 group (vehicle), not detectable (≤0.05 ng/ml); 0.6 ng/ml group, 0.480 ± 0.021 ng/ml; and 2.0 ng/ml group, 1.93 ± 0.048 ng/ml.

Table 5. Means (± SEM) for the Exploration Times and d2 Index in the Natural Forgetting ORT after Six-Day Continuous EVP-6124 Treatment

Targeted plasma C_{ss} of EVP-6124 (ng/ml)	e1 (sec)	e2 (sec)	d2 index
0.0	27.10 (2.40)	29.54 (1.88)	-0.02 (0.05)
0.6	22.40 (1.33)	27.86 (1.64)	0.33 (0.07)$^{###}$
2.0	21.66 (1.84)	28.80 (1.50)	0.19 (0.04)$^{###}$

Dose was based on the weight of the free base. The d2 index differed from zero by one-sample *t*-tests: $^{###}P$<0.001.

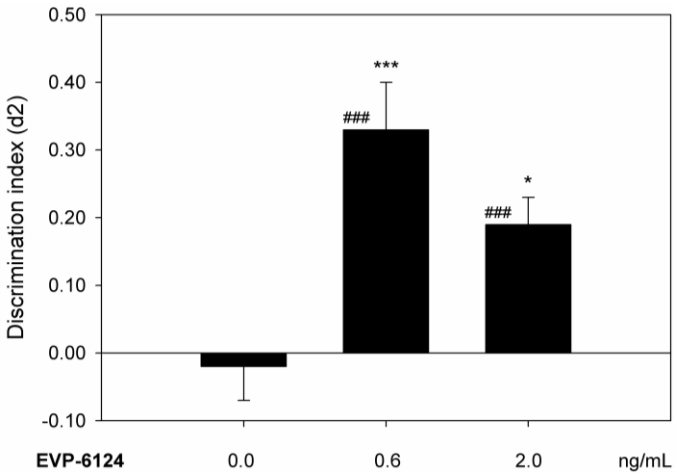


Figure 2. Mean d2 index in the natural forgetting ORT after six-day continuous EVP-6124 treatment. When compared with the vehicle group (0.0, EVP-6124), C_{ss} of both 0.6 and 2.0 ng/ml of EVP-6124 prevented natural forgetting. A significant post-hoc difference from the vehicle group, following one-way ANOVA: **P*<0.05, ****P*<0.001. A significant difference from zero in one-sample *t*-tests: $^{###}P$<0.001). n = 16 animals per group. Error bars represent SEM.

Discussion

The identification of a high expression level of homopentameric α7 nAChRs in brain areas like the hippocampus and cortex, suggested that these receptors may play an important role in cognitive functions (Dani and Bertrand, 2007). This hypothesis was confirmed by the finding that specific agonists of α7 nAChRs improved memory performance in animals, as shown initially by the effects of molecules such as GTS-21 (reviewed in: Kem, 2000) and more recently by more selective and potent agonists or modulators (e.g. reviewed in: Wallace and Porter, 2011; Posadas et al., 2013).

The cognitive enhancing effects of the partial α7 nAChR agonist EVP-6124 on natural forgetting (i.e., after a 24-h inter-trial interval) in the ORT were assessed in this study. In particular, we investigated whether this procognitive effect of EVP-6124 could be observed after six days of continuous EVP-6124 treatment by means of minipump administration. If this procognitive effect was still observed after six days of continuous EVP-6124 exposure, this would suggest a lack of tolerance development to the cognition-enhancing effects of this selective α7 nAChR agonist. In our previous study, EVP-6124 demonstrated good brain

penetration after oral administration, with brain:plasma ratios of approximately 2 between 1 and 4 h post-dosing (Prickaerts et al., 2012).

In the vehicle groups, the rats, as expected, did not remember the familiar object after a 24-h interval. A single 0.3 mg/kg, p.o. dose of EVP-6124 completely prevented natural forgetting of the familiar object. An intermediate prevention of natural forgetting was observed after a single 1.0 mg/kg oral dose of EVP-6124 (i.e., the group differed in the one-sample *t*-test but not in the comparison with the vehicle group). In this acute single-oral dose study, none of the doses of EVP-6124 had an effect on the exploratory activity of the animals, indicating that EVP-6124 did not affect exploratory behavior and/or (locomotor) activity per se. Although, conclusions regarding (locomotor) activity have to be drawn with caution considering that this was not the main objective to measure in the ORT. Still no indications for affected locomotor activity were observed. Targeted EVP-6124 plasma C_{ss} values on day 6 of 0.6 (measured plasma C_{ss} of 0.480 ng/ml) and 2.0 ng/ml (measured plasma C_{ss} of 1.93 ng/ml), maintained for six days by minipump administration, also prevented natural forgetting of the familiar object. Also in this minipump study, the targeted C_{ss} EVP-6124 values had no effect on the exploratory activity of the animals.

In the present single-dose study, 1.0 mg/kg, p.o. EVP-6124 produced less memory enhancement than 0.3 mg/kg, p.o. EVP-6124, an effect that may be attributed to overstimulation of the α7 nAChRs, although other (side) effects may not be ruled out. The data from the continuous exposure experiment showed that both plasma C_{ss} values of 0.48 ng/ml (targeted 0.6 ng/ml C_{ss} group) and 1.93 ng/ml (targeted 2.0 ng/ml C_{ss} group) significantly improved memory (i.e. prevented natural forgetting) in the ORT, suggesting a lack of tolerance development to the cognition-enhancing properties of EVP-6124 for at least 6 days in these rats. While there were no apparent statistically significant differences between the 0.48 and 1.93 ng/ml C_{ss} conditions, the shape of the graph is very similar between the acute-single and continuous dosing experiments (see Figures 1 and 2), i.e. suggestive of an inverted-U-shaped dose-response curve in both experiments. Of note, the plasma concentration of EVP-6124 after a single dose of 0.3 mg/kg, p.o. was approximately 0.3 ng/ml at 1-4 h post-dosing in the rat (Prickaerts et al., 2012). These data indicate that the plasma concentrations required for efficacy after a single-oral dose are similar to those required under steady state conditions.

In the proof-of-concept, randomized trial of EVP-6124 in patients with schizophrenia, EVP-6124 appeared to have good tolerability and safety at doses

of 0.3 and 1.0 mg/day for up to 21 days. After 21 days of EVP-6124 treatment in patients with schizophrenia on stable antipsychotic treatment, positive effects were found on measures of cognitive functioning. In addition, significant effects were found on electroencephalography (EEG) indicators of brain function (Preskorn et al., 2014). This also suggests a lack of tolerance development after 21 days of treatment in these patients.

Speculating about this lack of tolerance after continuous administration of EVP-6124 in both rats and humans, no signs of behavioral desensitization (i.e. tolerance) to the cognition-enhancing effects of the compound seem to develop. This could suggest that at cognition-enhancing doses, no functional desensitization of the α7 nAChRs occurred. However, this statement has to be interpreted with great caution since no *in vitro* desensitization studies were conducted in this study. Future studies should evaluate these desensitization features in order to investigate these statements. Nevertheless, from these data, we can conclude that at memory enhancing doses, no behavioral desensitization seems to develop.

In the single-dose study, EVP-6124 (0.3 mg/kg, p.o.) was administered 30 min before T1 of the ORT. Considering the total plasma concentration remained approximately 0.3 ng/ml for at least 4 h post-dosing (Prickaerts et al., 2012), the memory enhancing effects seen in these studies could have been due to a positive effect of EVP-6124 on memory acquisition, as well as on memory consolidation (van Goethem et al., 2012). In our previous EVP-6124 study (Prickaerts et al., 2012), we have shown that EVP-6124 also could improve memory consolidation (i.e. when administered immediately after T1 in the ORT). Similar effects have been found with the partial α7 nAChR agonist RG3487, which significantly improved both memory acquisition (administered p.o. 1 h before the learning trial) and memory consolidation (administered i.p. immediately after the learning trial) in rats (Wallace et al., 2011). In the continuous administration study, EVP-6124 was present at all phases of memory (acquisition, consolidation and retrieval). In spite of the continuous exposure to EVP-6124, the maximum drug effects on memory with a single dose of 0.3 mg/kg, p.o. and continuous administration to a C_{ss} of 0.6 ng/ml were similar, suggesting that a ceiling effect for improvement in cognition was met.

Many α7 nAChR agonists show affinity for the serotonin 5-HT_3 receptor, on which they act as an antagonist (e.g. Boess et al., 2007; Wallace et al., 2011). The 5-HT_3 receptor is structurally similar to the α7 nAChR and as such embodies another member of the super family of ligand-gated ion channels to which the

α7 nAChR belongs. The binding profile of EVP-6124 shows high-affinity antagonism for the 5-HT_3 receptor as well (Prickaerts et al., 2012). It has been suggested that the enhancement seen in cognitive tasks after administration of α7 nAChR agonists, may be (partly) mediated via the 5-HT_3 mechanism (for a review, see: Walstab et al., 2010). However, a broad dose range of ondansetron (0.1-10 mg/kg, p.o.), a selective 5-HT_3 receptor antagonist, did not enhance memory in a natural forgetting test of the ORT in rats. On the other hand, the α7 nAChR agonist and 5-HT_3 receptor antagonist RG3487 was effective in this test (Wallace et al., 2011). Furthermore, the brain concentrations of EVP-6124 and other α7 nAChR agonists required for efficacy in cognitive tests (sub- to low nanomolar range) are below the concentrations required for antagonist activity at 5-HT_3 receptors where near to full receptor occupancy would likely be required (Prickaerts et al., 2012).

In conclusion, EVP-6124 prevented natural forgetting after an inter-trial interval of 24 h in the ORT after a single dose of 0.3 mg/kg, p.o. in male Wistar rats. When measured plasma C_{ss} values of 0.48 and 1.93 ng/ml of EVP-6124 were maintained for six days by minipump administration, natural forgetting was also prevented. Comparable plasma concentrations were required in the single-dose and continuous infusion studies for optimal prevention of natural forgetting. Therefore, these data showed a lack of tolerance development after continuous administration of the α7 nAChR agonist EVP-6124 at memory enhancing doses in rats.

Acknowledgements
PK analysis was performed by Agilux Laboratories (Worcester, MA).

References

Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., Rogers, S. W. (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. *Physiological Reviews, 89*(1), 73-120.

Bitner, R. S., Bunnelle, W. H., Decker, M. W., Drescher, K. U., Kohlhaas, K. L., Markosyan, S., et al. (2010). In vivo pharmacological characterization of a novel selective α7 neuronal nicotinic acetylcholine receptor agonist ABT-107: preclinical considerations in Alzheimer's disease. *Journal of Pharmacology and Experimental Therapeutics, 334*(3), 875-886.

Boess, F. G., De Vry, J., Erb, C., Flessner, T., Hendrix, M., Luithle, J., et al. (2007). The novel α7 nicotinic acetylcholine receptor agonist *N*-[(3*R*)-1-azabicyclo [2.2.2] oct-3-yl]-7-[2-(methoxy) phenyl]-1-benzofuran-2-carboxamide improves working and recognition memory in rodents. *Journal of Pharmacology and Experimental Therapeutics, 321*(2), 716-725.

Dani, J. A. (2001). Overview of nicotinic receptors and their roles in the central nervous system. *Biological Psychiatry, 49*(3), 166-174.

Dani, J. A., and Bertrand, D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews Pharmacology and Toxicololgy, 47*, 699-729.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31(1)*, 47-59.

Kem, W. R. (2000). The brain α7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). *Behavioural Brain Research, 113*(1-2), 169-181.

Levin, E. D., and Simon, B. B. (1998). Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology, 138*(3-4), 217-230.

Mansvelder, H. D., and McGehee, D. S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron, 27*(2), 349-357.

Newhouse, P. A., Potter, A., Levin, E. D. (1997). Nicotinic system involvement in Alzheimer's and Parkinson's diseases. Implications for therapeutics. *Drugs & Aging, 11*(3), 206-228.

Newhouse, P. A., Potter, A., Singh, A. (2004). Effects of nicotinic stimulation on cognitive performance. *Current Opinion in Pharmacology, 4*(1), 36-46.

Othman, A. A., Lenz, R. A., Zhang, J., Li, J., Awni, W. M., Dutta, S. (2011). Single- and multiple-dose pharmacokinetics, safety, and tolerability of the selective α7 neuronal nicotinic receptor agonist, ABT-107, in healthy human volunteers. *Journal of Clinical Pharmacology, 51*(4), 512-526.

Picciotto, M. R., Caldarone, B. J., King, S. L., Zachariou, V. (2000). Nicotinic receptors in the brain: links between molecular biology and behavior. *Neuropsychopharmacol*ogy*, 22*, 451-465.

Posadas, I., López-Hernández, B., Ceña, V. (2013). Nicotinic receptors in neurodegeneration. *Current Neuropharmacology, 11*(3), 298-314.

Preskorn, S. H., Gawryl, M., Dgetluck, N., Palfreyman, M., Bauer, L. O., Hilt, D. C. (2014). Normalizing effects of EVP-6124, an alpha-7 nicotinic partial agonist, on event-related potentials and cognition: a proof of concept, randomized trial in patients with schizophrenia. *Journal of Psychiatric Practice, 20*(1), 12-24.

Prickaerts, J., Steinbusch, H. W. M., Smits, J. F. M., de Vente, J. (1997). Possible role of nitric oxide-cyclic GMP pathway in object recognition memory: effects of 7-nitroindazole and zaprinast. *European Journal of Pharmacology, 337*(2-3), 125-136.

Prickaerts, J., van Goethem, N. P., Chesworth, R., Shapiro, G., Boess, F. G., Methfessel, C., et al. (2012). EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacoly, 62*(2), 1099-1110.

Siok, C. J., Rogers, J. A., Kocsis, B., Hajós, M. (2006). Activation of α7 acetylcholine receptors augments stimulation-induced hippocampal theta oscillation. *European Journal of Neuroscience, 23*(2), 570-574.

Toyohara, J., and Hashimoto, K. (2010). α7 nicotinic receptor agonists: potential therapeutic drugs for treatment of cognitive impairments in schizophrenia and Alzheimer's disease. *Open Medicinal Chemistry Journal, 4*, 37-56.

van Goethem, N. P., Rutten, K., van der Staay, F-J., Jans, L. A. W., Akkerman, S., Steinbusch, H. W. M., et al. (2012). Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research, 232*(2), 323-334.

Wallace, T. L., Callahan, P. M., Tehim, A., Bertrand, D., Tombaugh, G., Wang, S., et al. (2011). RG3487, a novel nicotinic α7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. *Journal of Pharmacology and Experimental Therapeutics, 336*(1), 242-253.

Wallace, T. L., and Porter, R. H. P. (2011). Targeting the nicotinic alpha7 acetylcholine receptor to enhance cogntion in disease. *Biochemical Pharmacoly, 82*(8), 891-903.

Walstab, J., Rappold, G., Niesler, B. (2010). 5-HT_3 receptors: role in disease and target of drugs. *Pharmacology & Therapeutics, 128*(1), 146-169.

Chapter 7

Donepezil and the α7 agonist PHA 568487, but not risperidone, ameliorate spatial memory deficits in a subchronic MK-801 mouse model of cognitive impairment in schizophrenia

Stoyo Karamihalev, Jos Prickaerts and Nick P. van Goethem
Behavioural Brain Research (2014), 272(1), 248-251

Abstract
Cognitive impairment associated with schizophrenia (CIAS) is an important etiological feature of this disorder with implications for symptom severity and quality of life. Acute N-methyl-D-aspartate receptor (NMDAR) blockade using MK-801, a non-competitive antagonist to NMDARs, is assumed to produce temporary cognitive impairments in mice similar to those seen in schizophrenic patients. Less is known, however, about the effects of subchronic MK-801 administration on cognition. In the current study, twenty-eight male C57/BL6 mice received a daily dose of MK-801 (0.1 mg/kg, i.p.) for seven days. Spatial memory was assessed using an object location task prior to MK-801 administration as well as at multiple time points after the treatment. Subchronic treatment with MK-801 caused lasting memory deficits, which were ameliorated by acute doses of an acetylcholinesterase inhibitor (donepezil) and an α7 nAChR agonist (PHA 568487), but were unaffected by acute administration of the atypical antipsychotic risperidone. Subchronic administration of MK-801 may lend this pharmaceutical model increased face validity, while its resemblance to prodromal schizophrenia makes it suitable for screening new CIAS treatments.

Glutamatergic hypofunction is a well-established feature of schizophrenia and has been used extensively in modelling cognitive impairment associated with schizophrenia (CIAS) (Javitt, 2012; Meltzer et al., 2013). Acute treatment with MK-801, a non-competitive N-methyl-D-aspartate receptor (NMDAR) antagonist, is a common way of creating schizophrenia-like cognitive deficits in rodents (van der Staay et al., 2011). This model has been shown to possess reasonable predictive validity, with no responses to antipsychotics (e.g. risperidone, clozapine) and positive responses to putative cognitive enhancers (e.g. nicotine) mimicking those of human patients (Brown et al., 2014). As such, the acetylcholinesterase inhibitor donepezil, which is used as a cognition enhancer, consistently reversed the cognitive deficits induced by an acute dose of MK-801 (Csernansky et al., 2005; Brown et al., 2014), while it has not been successful in improving human CIAS (Keefe et al., 2008; Thakurathi et al., 2013).

As schizophrenia is a chronic disorder, (sub)chronic treatment with MK-801 might resemble CIAS better. However, less is known about the consequences of subchronic MK-801 administration in rodents. Reports of enduring effects of subchronic MK-801 regimens include locomotor changes (Farley et al., 2012), changes in affect (increased immobility in the forced swim paradigm) (Langen et al., 2012), and memory performance (in the object recognition test) (Bado et al., 2011). Prevention of memory impairments resulting from subchronic NMDA receptor antagonist treatment in the latter task has been suggested to translate as treatment against CIAS in the clinic (Rajagopal et al., 2013).

In order to explore further the suitability of subchronic MK-801 administration as a preclinical model for testing possible CIAS treatments in rodents, we used a spatial variant of the object recognition task, i.e. the object location task (OLT). We assessed spatial memory prior to, as well as at multiple time points following subchronic MK-801 treatment in mice. In addition, the effects on memory performance of the atypical antipsychotic risperidone and the putative cognition-enhancers donepezil and PHA 568487 were tested. PHA 568487 is an agonist of the alpha7 nicotinic acetylcholine receptor (α7 nAChR). α7 nAChR agonists have shown positive effects in counteracting acute MK-801-induced memory deficits (Pichat et al., 2007; Haydar et al., 2009) and are currently considered a promising treatment option for CIAS (Toyohara and Hashimoto, 2010).

The OLT was performed as described in Bruno et al. (2011). Briefly, in a first learning trial a mouse is exposed to an arena with two identical objects. Following a one hour interval, the mouse is placed into the same arena, but with one of the objects placed at a new location. Exploration time for each object is

recorded. The preference for the object at the new location in the second trial is considered representative of spatial memory capacity (Murai et al., 2007). The main outcome measure is a discrimination index (d2): the difference in exploration time between the displaced and stationary object, divided by the total exploration time for the trial. D2 is a relative measure of discrimination, independent of exploratory activity. A positive d2, significantly different from zero, is considered indicative of successful recall of spatial information (tested using a one-sample *t*-test (Akkerman et al., 2012[A])). All animals were trained until they showed a stable performance at a one hour interval. Comparisons between treatments were done using one-way analyses of variance (ANOVAs) followed by LSD post hoc tests.

A low dose of MK-801 (Research Biochemicals International/Sigma-Aldrich, Deisenhofen, Germany) was administered daily (0.1mg/kg dissolved in saline, i.p.) to twenty-eight seven month-old male C57/BL6 mice (Charles River, Sulzfeld, Germany) for seven days. All mice were housed individually in standard Tecniplast IVC system greenline cages, on sawdust bedding, with a reversed light/dark cycle of 12/12 hours (lights off from 7:00 to 19:00). Baseline OLT scores were obtained prior to the subchronic MK-801 regimen. Drug tests were conducted after a seven day washout period. Thirty minutes before the first OLT trial, mice received oral injections of either vehicle (saline; n=9), donepezil (1mg/kg dissolved in saline, n=10), or risperidone (0.1mg/kg dissolved in 98% tylose solution (0.5%) with 2% tween80, n=9). Injection volume was 2.0 ml/kg. Donepezil and risperidone were a kind gift of Abbott (Weesp, The Netherlands). Both doses were selected as they are known to improve memory (donepezil) and prepulse inhibition performance (risperidone) in rodents (Csernansky et al., 2005; Bubenikova-Valesova et al., 2008; Brown et al., 2014). Since the same group of mice was tested multiple times, allocation to treatment conditions was randomized for the first test and counterbalanced for further tests (see below), such that each mouse was assigned to the vehicle group once for one of three tests. Exploration times were scored manually by an experimenter unaware of the treatment conditions being tested.

Following subchronic MK-801 administration, the vehicle treated group did not show a preference for the displaced object (d2 not different from zero; $t_8 = 1.31$, $P = 0.227$), suggesting memory impairment associated with MK-801 exposure (Fig.1). This was further supported by a paired samples *t*-test, which revealed a significant decrease in d2 from the pre-MK-801 treatment baseline for the vehicle treated animals (t_8=2.332, $P < 0.05$]. The one-way ANOVA showed no treatment effect ($F_{2, 25} = 1.149$, ns], however the memory deficit was partly ameliorated by a single dose of donepezil as donepezil-treated mice showed a

preference for the displaced object (comparison with zero using a one-sample *t*-test: t_9 = 3.144, *P* = 0.012). The d2 score of the risperidone group was not different from zero (t_8 = .939, P = 0.375), indicating that risperidone had no effect at all on spatial memory at this dose.

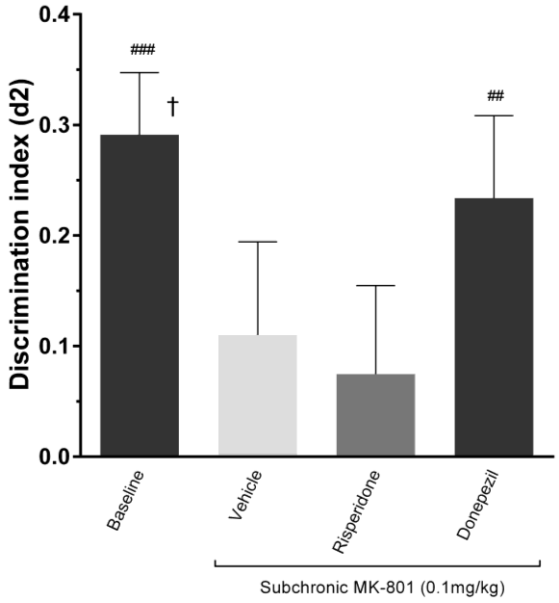


Figure 1. Effects of risperidone (0.1mg/kg) and donepezil (1mg/kg) on MK-801-induced spatial memory deficits measured by the discrimination index (d2) of the object location task at baseline (n = 28) and at seven days after subchronic MK-801 (0.1mg/kg; injected daily for seven days) treatment (means ± SEM). A difference from zero is represented by hashes (one-sample *t*-tests: ## p<0.01; ###p<0.001). †p<0.05 difference between baseline and vehicle (paired-samples *t*-test).

Twenty-four days after the end of the subchronic MK-801 treatment, mice were tested again on the OLT thirty minutes after receiving oral injections of either saline (n=9), or one of two doses of the α7 nAChR agonist PHA 568487 (0.3 mg/kg, n=10; 1mg/kg, n=9; PHA 568487 was a kind gift from Forum pharmaceuticals, Boston, USA), dissolved in saline in an injection volume of 2.0 ml/kg. The same test was conducted five days later, i.e. twenty-eight days after the end of the subchronic MK-801 treatment, with a saline treated (n=9) group and two additional groups treated with lower doses of the α7 nAChR agonist (0.03mg/kg, n=9; 0.01mg/kg, n=10).

At both time points d2 remained non-significantly different from zero for the vehicle groups (t_8 = 1.18, *P* = 0.272; and t_8 = 0.109, *P* = 0.916 respectively), and paired-samples *t*-tests revealed significant d2 reductions from baseline for each vehicle condition (t_8 = 2.637, *P* = 0.03; and t_8 = 3.145, *P* = 0.014, respectively). This indicates clear memory impairment at both time points. Testing d2 scores in the treatment groups against zero showed that PHA 568487 ameliorated the

MK-801-induced memory deficit at doses of 0.1mg/kg (t_9 = 4.65, P = 0.001), 0.3mg/kg (t_9 = 5.421, P < 0.001), and 1mg/kg (t_8 = 4.22, P = 0.003), but not at the lowest dose of 0.03mg/kg (t_8 = 1.913, P = 0.092). One-way ANOVAs revealed significant treatment effects on both test days ($F_{2, 25}$ = 4.013, P = 0.031; and $F_{2, 25}$ = 3.639, P = 0.041 respectively). Post hoc LSD comparisons showed significant differences between the treatment and the local vehicle condition at doses of and 0.1 mg/kg (P = 0.013), 0.3 mg/kg (P = 0.021), and 1mg/kg (P = 0.02), suggesting notable memory improvement at these doses (Akkerman et al., 2012[B]) (Fig 2).

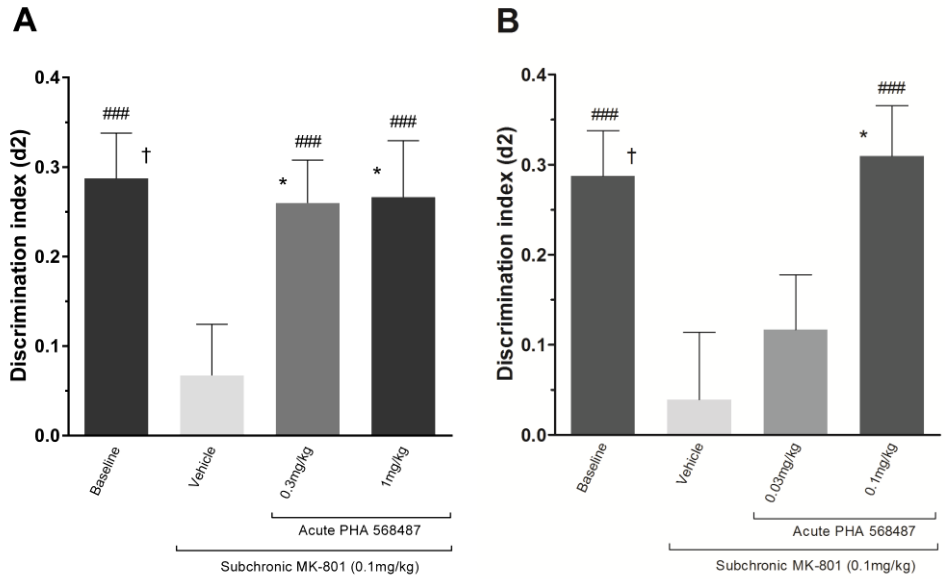


Figure 2. A) Effects of the α7 nAChR agonist PHA 568487 on MK-801-induced spatial memory deficits measured by the discrimination index (d2) of the object location task at baseline (n = 28) and on *day 24* after seven day subchronic MK-801 (0.1mg/kg) treatment (means ± SEM). **B)** Effects of the α7 nAChR agonist PHA 568487 on MK-801-induced spatial memory deficits measured by the discrimination index (d2) of the object location task at baseline (n = 28) and on *day 28* after seven day subchronic MK-801 (0.1mg/kg) treatment (mean ± SEM). Difference from zero is represented by hashes (one-sample *t*-tests: ##p<0.01; ###p<0.001). Difference from the local vehicle condition is represented by asterisks (Post-hoc LSD pairwise comparisons: *p<0.05). **†**p<0.05 difference between baseline and vehicle (paired-samples *t*-test).

In summary, low-dose subchronic MK-801 administration induced a lasting memory deficit in mice, which persisted at least 28 days after the last drug administration. This deficit was ameliorated by donepezil and an agonist to the α7 nAChR. The atypical antipsychotic risperidone was not effective at the dose

of 0.1mg/kg. The predictive validity of the subchronic MK-801 model of CIAS is subject to the same limitation as the acute model, as revealed by the false positive response of mice to donepezil (Brown et al., 2014). Drug responses of the animals in the subchronic MK-801 model are in line with findings from acute MK-801 exposure studies, but represent an extension of the latter, since subchronic MK-801 administration resulted in enduring memory deficits, lending this model increased face validity at a functional level.

The current study and previous research has demonstrated that subchronic MK-801 administration can produce behaviors resembling the cognitive and the negative symptoms of schizophrenia, but, to our knowledge, no evidence has been published showing that subchronic MK-801 treatment can produce positive-symptom-like behaviors in rodents (e.g. reduced prepulse inhibition of startle or locomotor hyperactivity (van den Buuse, 2010)). Some researchers have reported indications of locomotor reduction in rodents subchronically treated with MK-801 (Ashby et al., 2010), while others have found no effects (Beninger et al., 2009). The design of the current study does not allow for claims regarding the effect of subchronic MK-801 on locomotor activity. Exploration times were, however, sufficient in order to draw reliable conclusions about object location discrimination (Table 1) (Akkerman et al., 2012[B]). The ostensible absence of behaviors associated with positive symptoms (Beninger et al., 2009; Ashby et al., 2010) suggests a behavioral phenotype akin to the prodromal stages of schizophrenia, which include cognitive disturbances and early signs of negative symptomatology, but no positive symptoms (Phillips and Seidman, 2008; Kahn and Keefe, 2013). The apparent lack of increased exploration which would have indicated hyperlocomotor effects, has the added methodological benefit of removing a behavioral confound (Gilmour et al., 2012).

At this stage, subchronic MK-801 exposure does not appear to be an improvement on the drug screening capacities of the acute MK-801 model of CIAS, but its enduring effects carry the added benefit of a closer resemblance to the cognitive deficits seen in human schizophrenic patients. Future studies should further investigate the validity of this model by examining the type of damage caused by subchronic MK-801 administration and its psychotomimetic properties.

Table 1. Trial 1 OLT exploration times in seconds by test number and treatment condition.

Test	Group	Exploration Time (s)	
		Mean	SD
Baseline	all	34.0	10.8
Test 1	vehicle	24.3	8.6
	risperidone (0.1mg/kg)	15.1	5.8
	donepezil (1mg/kg)	25.0	10.4
Test 2	vehicle	25.9	7.5
	PHA 568487 (0.3mg/kg)	30.2	10.2
	PHA 568487 (1mg/kg)	29.9	9.9
Test 3	vehicle	22.1	6.8
	PHA 568487 (0.03mg/kg)	17.4	8.2
	PHA 568487 (0.1mg/kg)	22.5	8.6

Acknowledgements
The authors would like to thank Thecla van Wageningen for her technical assistance.

References

Akkerman, S., Prickaerts, J., Steinbusch, H. W. M., Blokland, A. (2012a). Object recognition testing: statistical considerations. *Behavioural Brain Research, 232*(2), 317-322.

Akkerman, S., Blokland, A., Reneerkens, O., van Goethem, N. P., Bollen, E., Gijselaers, H. J. M., et al. (2012b). Object recognition testing: methodological considerations on exploration and discrimination measures. *Behavioural Brain Research, 232*(2), 335-347.

Ashby, D. M., Habib, D., Dringenberg, H. C., Reynolds, J. N., Beninger, R. J. (2010). Subchronic MK-801 treatment and post-weaning social isolation in rats: differential effects on locomotor activity and hippocampal long-term potentiation. *Behavioural Brain Research, 212*(1), 64-70.

Bado, P., Madeira, C., Vargas-Lopes, C., Moulin, T. C., Wasilewska-Sampaio, A. P., Maretti, L., et al. (2011). Effects of low-dose D-serine on recognition and working memory in mice. *Psychopharmacology, 218*(3), 461-470.

Beninger, R. J., Forsyth, J. K., van Adel, M., Reynolds, J. N., Boegman, R. J., Jhamandas, K. (2009). Subchronic MK-801 behavioural deficits in rats: partial reversal by the novel nitrate GT 1061. Pharmacology, *Biochemistry and Behavior, 91*(4), 495-502.

Brown, J. W., Rueter, L. E., Zhang, M. (2014). Predictive validity of a MK-801-induced cognitive impairment model in mice: implications on the potential limitations and challenges of modeling cognitive impairment associated with schizophrenia preclinically. *Progress in Neuro-psychopharmacology and Biological Psychiatry, 49*, 53-62.

Bruno, O., Fedele, E., Prickaerts, J., Parker, L., Canepa, E., Brullo, C., et al. (2011). GEBR-7b, a novel PDE4D selective inhibitor that improves memory in rodents at non-emetic doses. *British Journal of Pharmacology, 164*(8), 2054-2063.

Bubenikova-Valesova, V., Stuchlik, A., Svoboda, J., Bures, J., Vales, K. (2008). Risperidone and ritanserin but not haloperidol block effect of dizocilpine on the active allothetic place avoidance task. *Proceedings of the National Academy of Sciences of the Unisted States of America, 105*(3), 1061-1066.

Csernansky, J. G., Martin, M., Shah, R., Bertchume, A., Colvin, J., Dong, H. (2005). Cholinesterase inhibitors ameliorate behavioral deficits induced by MK-801 in mice. *Neuropsychopharmacology, 30*, 2135-2143.

Farley, S., Dumas, S., El Mestikawy, S., Giros, B. (2012). Increased expression of the Vesicular Glutamate Transporter-1 (VGLUT1) in the prefrontal cortex correlates with differential vulnerability to chronic stress in various mouse strains: effects of fluoxetine and MK-801. *Neuropharmacology, 62*(1), 503-517.

Gilmour, G., Dix, S., Fellini, L., Gastambide, F., Plath, N., Steckler, T., et al. (2012). NMDA receptors, cognition and schizophrenia--testing the validity of the NMDA receptor hypofunction hypothesis. *Neuropharmacology, 62*(3), 1401-1412.

Haydar, S. N., Ghiron, C., Bettinetti, L., Bothmann, H., Comery, T., Dunlop, J., et al. (2009). SAR and biological evaluation of SEN12333/WAY-317538: Novel alpha 7 nicotinic acetylcholine receptor agonist. *Bioorganic and Medicinal Chemistry, 17*(14), 5247-5258.

Javitt, D. C. (2012). Twenty-five years of glutamate in schizophrenia: are we there yet? *Schizophrenia Bulletin, 38*(5), 911-913.

Kahn, R. S., Keefe, R. S. E. (2013). Schizophrenia is a cognitive illness: time for a change in focus. *JAMA Psychiatry, 70*(10), 1107-1112.

Keefe, R. S. E., Malhotra, A. K., Meltzer, H. Y., Kane, J. M., Buchanan, R. W., Murthy, A., et al. (2008). Efficacy and safety of donepezil in patients with schizophrenia or schizoaffective disorder: significant placebo/practice effects in a 12-week, randomized, double-blind, placebo-controlled trial. *Neuropsychopharmacology, 33*, 1217-1228.

Langen, B., Dost, R., Egerland, U., Stange, H., Hoefgen, N. (2012). Effect of PDE10A inhibitors on MK-801-induced immobility in the forced swim test. *Psychopharmacology, 221*(2), 249-259.

Meltzer, H.Y., Rajagopal, L., Huang, M., Oyamada, Y., Kwon, S., Horiguchi, M. (2013). Translating the N-methyl-D-aspartate receptor antagonist model of schizophrenia to treatments for cognitive impairment in schizophrenia. *International Journal of Neuropsychopharmacology, 16*(10), 2181-2194.

Murai, T., Okuda, S., Tanaka, T., Ohta, H. (2007). Characteristics of object location memory in mice: Behavioral and pharmacological studies. *Physiology & Behavior, 90*(1), 116–124.

Phillips, L. K., Seidman, L. J. (2008). Emotion processing in persons at risk for schizophrenia. *Schizophrenia Bulletin, 34*(5), 888-903.

Pichat, P., Bergis, O. E., Terranova, J-P., Urani, A., Duarte, C., Santucci, V., et al. (2007). SSR180711, a novel selective alpha7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. *Neuropsychopharmacology, 32*, 17-34.

Rajagopal, L., Massey, B. W., Huang, M., Oyamada, Y., Meltzer, H. Y. (2013). The Novel Object Recogniton Test in Rodents in Relation to Cognitive Impairment in Schizophrenia. *Current Pharmaceutical Design, 20*(31), 5104-5114.

Thakurathi, N., Vincenzi, B., Henderson, D. C. (2013). Assessing the prospect of donepezil in improving cognitive impairment in patients with schizophrenia. *Expert Opinion on Investigational Drugs, 22*(2), 259-265.

Toyohara, J., Hashimoto, K. (2010). α7 Nicotinic Receptor Agonists: Potential Therapeutic Drugs for Treatment of Cognitive Impairments in Schizophrenia and Alzheimer's Disease. *The Open Medicinal Chemistry Journal, 4*, 37-56.

van den Buuse, M. (2010). Modeling the positive symptoms of schizophrenia in genetically modified mice: pharmacology and methodology aspects. *Schizophrenia Bulletin, 36*(2), 246-270.

van der Staay, F. J., Rutten, K., Erb, C., Blokland, A. (2011). Effects of the cognition impairer MK-801 on learning and memory in mice and rats. *Behavioural Brain Research, 220*(1), 15-29.

Chapter 8

Blocking α7 nicotinic acetylcholine receptors improves specifically memory acquisition

Nick P. van Goethem[1], Ernesto Fedele[1], Daniela Puzzo[1], Claudia Rebosio, Walter Gulisano, Agostino Palmeri, Lawrence P. Wennogle, Youyi Peng, Harry W.M. Steinbusch and Jos Prickaerts

Submitted to Neuropsychopharmacology

Abstract

α7 nicotinic acetylcholine receptors (α7 nAChRs) are ligand-gated ion channels expressed primarily in the brain. These receptors have been implicated in modulating cognitive functions like episodic memory and attention. Hence, α7 nAChR agonists/modulators may be attractive drug candidates to improve cognition in Alzheimer's disease (AD) and schizophrenia. In the current study, the cognition enhancing properties of low dose administration of selective α7 nAChR antagonists were investigated in rats as low doses of methyllycaconitine (MLA) have sporadically been reported to improve cognition in animals. Memory acquisition and consolidation processes were assessed separately with the object recognition task (ORT). The compounds used for these studies were MLA and Compound 7i. Interestingly, it was found that low doses of MLA and Compound 7i improved the acquisition, but not the consolidation processes of object recognition memory at a 24 h retention interval. Conversely, higher doses impaired the memory performance at a shorter 1 h retention interval. In addition, the same compounds were studied in a model of neuronal plasticity, long-term potentiation (LTP). It was demonstrated that pre-tetanus low-dose administration of MLA or Compound 7i produced a longer lasting potentiation, whereas post-tetanus administration had no effect. Microdialysis studies showed that MLA administration substantially increased hippocampal glutamate efflux which has been found to be related to object memory processes. In summary, blocking α7 nAChRs with low doses of selective antagonists improves specifically the memory acquisition process. While the main focus of the α7 nAChR as a target for cognition enhancement lies on agonists and positive modulators, antagonism of these receptors at low doses might also prove to be a valuable tool for cognition enhancement in AD or schizophrenia.

Introduction

α7 nicotinic acetylcholine receptors (α7 nAChRs) are Ca^{2+} permeable ligand-gated ion channels expressed primarily in the brain. α7 nAChRs are homomeric receptors constituted by five identical transmembrane α7-subunits surrounding a central channel. These receptors have been implicated in modulating cognitive functions like attention and episodic memory (e.g. Toyohara and Hashimoto, 2010). α7 nAChRs are located pre-, post- and non-synaptically (Dani and Bertrand, 2006) and modulate the release of glutamate (Dickinson et al., 2008; Molas and Dierssen, 2014), GABA (Arias et al., 2010) and dopamine (Quarta et al., 2009). Furthermore, α7 nAChRs are directly involved in hippocampal long-term potentiation (LTP) (Mansvelder and McGehee, 2000), the putative cellular mechanism underlying learning and memory (Bliss and Collingridge, 1993). LTP can be divided into 2 phases; the transient early phase of LTP (E-LTP) and the long-lasting form which is called late phase LTP (L-LTP). This latter is dependent on protein synthesis and induces longer lasting plastic changes via, for instance cAMP-Responsive Elemen Binding (CREB) phosphorylation (Kandel, 2012). Administration of α7 nAChR ligands has been shown to improve cognitive function in both animal (Levin and Simon, 1998) and human studies (Newhouse et al., 1997, 2004). The main cognitive improvement with these compounds relate to memory, in accordance with the high level of expression of α7 nAChRs in the frontal-cortex and hippocampus. Different α7 nAChR agonists have been investigated for their potential to improve memory and attention disorders encountered for instance in Alzheimer's disease (AD) and schizophrenia (Toyohara and Hashimoto, 2010). A major drawback of α7 nAChRs is that they show rapid desensitization following exposure to agonists (Picciotto et al., 2000).

To possibly circumvent the desensitization issue associated with α7 nAChR agonists, the objective of the current study was to investigate the effects of low dose administration of selective α7 nAChR antagonists on memory function in rodents. Interestingly, low dose monotreatment with the α7 nAChR antagonist methyllycaconitine (MLA) has sporadically been reported to improve cognition in animals (Hahn et al., 2011; Burke et al., 2014). Furthermore, MLA has been shown to facilitate LTP induction in hippocampal region CA1 in rats (Fujii et al., 2000). The selective α7 nAChR antagonists used in the current studies were MLA and Compound 7i (Peng et al., 2010). MLA is a norditerpenoid alkaloid produced by many species of Delphinium (larkspurs) (Ward et al., 1990) and is selective for the α7 nAChR over other nAChRs or ligand-gated ion channels. MLA is often used because of its ability to potently antagonize the α7 nAChR in animal models to show that putative α7 nAChR agonists indeed work through the α7

nAChR mechanism (e.g. Prickaerts et al., 2012). Compound 7i is a compound originally developed as an antidote for organophosphorus nerve agent intoxication. It has been identified as an α7 nAChR antagonist with good brain penetration and selectivity for the neuronal α7 subtype over other nAChRs or ligand-gated ion channels (Peng et al., 2010).

In the present study, memory performance after α7 nAChR antagonist treatment was assessed with the object recognition task (ORT) in male rats. Both memory acquisition and consolidation processes were investigated separately within this paradigm. Furthermore, LTP electrophysiological studies with low and high dose administration (and pre- and post-tetanus schedule) of both compounds were performed. Since hippocampal glutamate efflux has been associated with object memory processes in rats (Stanley et al., 2012), microdialysis studies were also performed in which the glutamate and GABA efflux was measured in the hippocampus of male rats after administration of different doses of MLA.

Materials and methods

All procedures were designed to minimize the potential discomfort of the animals during the experiments. All experimental procedures were approved by the local ethical committees for animal experiments according to local governmental guidelines.

Reagents

Methyllycaconitine citrate salt hydrate (MLA) was obtained from Research Biochemicals International/Sigma-Aldrich (Deisenhofen, Germany). Compound 7i was a generous gift from Intra-Cellular Therapies, Inc. (New York, USA).

Animals

For the ORT studies, a total of thirty-six 3-4-months-old male Wistar rats (Charles River, Sulzfeld, Germany) were used. The animals were housed individually in temperature (21 ± 1°C)- and humidity (60 ± 10%)-controlled rooms; food and water were available *ad libitum*. The animals were maintained under a reversed 12/12-h light/dark cycle. During the dark period, fluorescent red tubes provided a constant illumination (2 lux). Behavioral experiments were performed in the dark period in the same room where the rats were housed. During testing the test room was dimly lit by a small lamp (25W), located in the corner of the room.

For the microdialysis studies, a total of twenty-one 3-month-old male Sprague Dawley (SD) rats (Charles River, Calco, Italy) were used. Animals were maintained on a 12 h light/dark cycle in temperature (21 ± 1°C)- and humidity (50%)-controlled rooms; food and water were available *ad libitum*.

For electrophysiological studies, we used a total of twenty-four C57BL/6J male mice, 3-5-month-old, obtained from a breeding colony housed in the animal facility of the University of Catania. Housing conditions of the mice were the same as for rats, except that they were housed socially with five animals per cage.

Object recognition task

The ORT training- and testing-procedures were performed as described elsewhere (Ennaceur and Delacour, 1988; van Goethem et al., 2012). The apparatus and objects were identical to those described previously (Akkerman et al., 2012). The same four objects were used during adaptation and in the studies. Because rats were retested with different compound doses, test sessions were scheduled to allow at least a two-day wash-out period.

Drug administration

MLA was prepared in saline at an injection volume of 1 ml/kg. Compound 7i was dissolved in a 1% tylose solution (98% of total end volume) with 2% tween80 at an injection volume of 1 ml/kg. The doses were based on the weight of the salts of the compounds. All solutions were prepared on the day of experimental testing and administered i.p. in a counterbalanced design.

ORT study designs

Memory acquisition experiments

The effect of MLA and Compound 7i on the acquisition process of long-term memory was investigated using a 24 h interval between the ORT learning- and test-trials (T1 and T2, respectively). The memory acquisition process was targeted by administrating the compounds before T1 in the ORT (van Goethem et al., 2012; Bollen et al., 2014). Untreated Wistar rats normally show no significant object memory performance after 24 h (e.g. Prickaerts et al., 2012). MLA (0.001 - 1.0 mg/kg, i.p.) was administered 30 min before T1. Compound 7i (0.003 - 3.0 mg/kg, i.p.) was administered 15 min before T1. The administration times of both compounds was chosen to correspond with the time for the compound to reach peak brain concentrations (MLA: Turek et al., 1995; Compound 7i: Peng et al., 2010). Both MLA and Compound 7i were also tested in a 1 h retention interval. Untreated Wistar rats normally show significant

object memory after a 1 h retention interval (e.g. Prickaerts et al., 2012). So, in this experiment the ability of the α7 nAChR antagonists to reverse significant memory using a 1 h retention interval was tested. Doses of 0.1 - 1.0 mg/kg MLA (i.p.) were administered 30 min before T1. Doses of 1.0 and 3.0 mg/kg Compound 7i (i.p.) were administered 15 min before T1. For the control treatment, animals received the vehicle of the respective drug. In both paradigms, all 18 animals received each treatment (within-subjects design).

Memory consolidation experiments

The effect of MLA and Compound 7i on the consolidation processes of long-term memory was also investigated using a 24 h interval between the ORT trials. The memory consolidation process was targeted by administrating the compounds after T1 in the ORT (van Goethem et al., 2012; Bollen et al., 2014). Optimum doses of MLA (0.03 - 0.1 mg/kg, i.p.) and Compound 7i (0.3 - 1.0 mg/kg, i.p.) were administered 4-10 min after T1 to specifically target memory consolidation processes (Bollen et al., 2014). The optimum doses of these compounds were based on the memory acquisition experiments. Like in the previous experiments, for the control treatment animals received the vehicle of the respective drug. All 18 animals received each treatment (within-subjects design).

Electrophysiology

Electrophysiological recordings were performed as previously described (Bollen et al., 2014). Briefly, transverse hippocampal slices (400 µm) were cut and transferred to a recording chamber where they were maintained at 29°C and perfused with ACSF (flow rate 2 ml/min) continuously bubbled with 95% O2 and 5% CO2. The ACSF composition was composed of the following (in mM): 124.0 NaCl, 4.4 KCl, 1.0 Na2HPO4, 25.0 NaHCO3, 2.0 CaCl2, 2.0 MgCl4, and 10.0 glucose. Field extracellular recordings were performed by stimulating the Schaeffer collateral fibers through a bipolar tungsten electrode and recording in CA1 stratum radiatum with a glass electrode filled with ACSF. A 15 min baseline was recorded every minute at an intensity that evoked a response approximately 35% of the maximum evoked response. Early-LTP was induced by a weak tetanus (4 pulses at 100 Hz, with the bursts repeated at 5 Hz and one tetanus of 10-burst trains) (Chapman et al., 1999; Zakharenko et al., 2003; Puzzo et al., 2009; Bollen et al., 2014; Ricciarelli et al., 2014). Compounds were administered for 10 min either right before or 10 min after tetanus to reflect behavioral treatment conditions (Bollen et al., 2014). Responses were recorded for 2 h after tetanization and measured as field excitatory post-synaptic

potentials (f-EPSP) slope expressed as a percentage of the baseline. The results were expressed as mean ± standard error mean.

Microdialysis

Rats were anaesthetized with chloral hydrate (400 mg/kg), placed in a stereotaxic frame (David Kopf Instruments, West Hempstead, NY, USA) and implanted with microdialysis probes which were transversely positioned into the dorsal hippocampi according to the following coordinates: AP = +3.8, H = +6.5 from the interaural line (Paxinos and Watson, 1986). A piece of dialysis fiber made of a co-polymer of acrylonitrile sodium methallyl sulphonate (AN69HF Hospal S.p.A., Bologna, Italy; 0.3 mm outer diameter, 40 kDa mol. wt. cut-off) was covered with epoxy glue to confine dialysis to the area of interest (8 mm glue-free lengths). The skull was exposed and two holes were drilled on the lateral surfaces. The dialysis probe, held straight by a tungsten wire inside, was inserted transversely into the brain so that the glue-free zone was exactly located into the target area. The tungsten wire was withdrawn and stainless steel cannulae (22-gauge diameter, 15-mm long) were glued to the ends of the fiber, bent up, and fixed vertically to the skull with dental cement and modified Eppendorf tips. After a 24-h recovery period, freely-moving rats were placed into observation cages and the probes infused at a flow rate of 5 µl/min (CMA/100 microinjection pump, CMA Microdialysis, Stockholm, Sweden) with modified Ringer's medium containing (in mM): NaCl 145, KCl 3, $MgCl_2$ 1, $CaCl_2$ 1.26, buffered at pH 7.4 with 2 mM phosphate buffer. Following a stabilization period of 1 h, consecutive samples were collected every 10 min. MLA was i.p. injected after 5 control samples had been collected to estimate basal glutamate and GABA levels. At the end of the experiment, rats were euthanized by anaesthetic overdose and the correct position of the probe was verified by optical examination of the fiber tract. Animals presenting hemorrhagic lesions or with a probe track outside the target region, were excluded from the results. Microdialysis samples were stored at -30 °C until HPLC analysis for amino acid determination as previously described (Cavallero et al., 2009).

Statistical analyses

The ORT provides measures for exploration time and discrimination (Akkerman et al., 2012). The basic measures are the times spent by the animals exploring an object during both the learning- and the test trial (T1 and T2, respectively). The times spent exploring the two identical objects in T1 will be represented by 'a1' and 'a2'. The times spent in exploring the familiar and the novel object in T2 will be represented by 'a3' and 'b', respectively. From these exploration times the following variables could be calculated: e1=a1+a2, e2=a3+b and d2=(b–a3)/e2.

e1 and e2 are measures of the total exploration time of both objects during T1 and T2, respectively. d2 is a relative measure of discrimination corrected for exploratory activity (e2). The d2 index can range from -1 to 1, with -1 or 1 indicating complete preference for the familiar or novel object, respectively and 0 signifying no preference for either object. In order to assess object recognition performance, one-sample *t*-statistics were performed. This way it could be assessed per treatment condition whether the d2 index differed significantly from zero (i.e. chance level). Effects on e1, e2 and d2 between the different treatment conditions were assessed by repeated measures ANOVA. When the overall ANOVA was significant, *post hoc* Bonferroni *t*-tests (all pairwise comparisons) were used. Animals with insufficient exploration times (< 7 s) were excluded from the dataset (Akkerman et al., 2012).

For the electrophysiological (LTP) recordings, statistical analysis was performed with two-way ANOVA with repeated measures. In the *in vivo* microdialysis study, all data were expressed as percentages of the mean basal value for each animal. The mean basal value has been calculated as the average of the first five microdialysis fractions before the injection (fraction 6). Data were analysed using one-way ANOVA. Significant main effects were followed by paired-samples *t*-tests between fraction 5 (the final baseline) and all subsequent collections. In case of a significant main effect, independent samples *t*-tests were also performed between the effective MLA dose and the vehicle/control group per fraction. In all analyses, an α level of 0.05 was considered significant.

Results

ORT: memory acquisition process: MLA

In the 24 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{7,119} = 1.84$; $P = 0.087$) or T2 (e2, $F_{7,119} = 1.59$; $P = 0.146$)**.** The d2 indices did differ between treatment conditions ($F_{7,119} = 4.37$; $P = 0.000$). Post hoc analyses revealed significantly higher object discrimination in the 0.003 mg/kg ($P = 0.041$), 0.03 mg/kg ($P = 0.020$) and 0.1 mg/kg MLA ($P = 0.002$) when compared to the vehicle condition. One-sample *t*-statistics showed that the discrimination performance was significantly different from chance level in the dosage range from 0.003 to 0.3 mg/kg MLA (Figure 1A). In the 1 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{3,51} = 0.27$; $P = 0.844$) or T2 (e2, $F_{3,51} = 0.90$; $P = 0.447$)**.** The d2 indices did differ between treatment conditions

($F_{3,51}$ = 7.33; P = 0.000). Post hoc analyses revealed significantly lower object discrimination in the 1.0 mg/kg MLA condition (P = 0.003) when compared to the vehicle condition. One-sample *t*-statistics showed that the discrimination performance was significantly different from zero in the vehicle, 0.1 and 0.3 mg/kg MLA conditions (Figure 1B).

ORT: memory acquisition process: Compound 7i

In the 24 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{5,80}$ = 0.03; P = 1.000) or T2 (e2, $F_{5,80}$ = 0.66; P = 0.657)**.** The d2 indices showed a tendency towards significant differences between treatment conditions ($F_{5,80}$ = 1.90; P = 0.104). One-sample *t*-statistics showed that the discrimination performance differed significantly from chance level in the dosage range from 0.1 to 1.0 mg/kg Compound 7i (Figure 1C). In the 1 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{2,34}$ = 0.87; P = 0.428) or T2 (e2, $F_{2,34}$ = 0.33; P = 0.719)**.** The d2 indices did differ between treatment conditions ($F_{2,34}$ = 16.21; P = 0.000). Post hoc analyses revealed significantly lower object discrimination in the 3.0 mg/kg Compound 7i condition (P = 0.000) when compared to the vehicle condition. One-sample *t*-statistics showed that the discrimination performance was significantly different from chance level in the vehicle and 1.0 mg/kg Compound 7i conditions (Figure 1D).

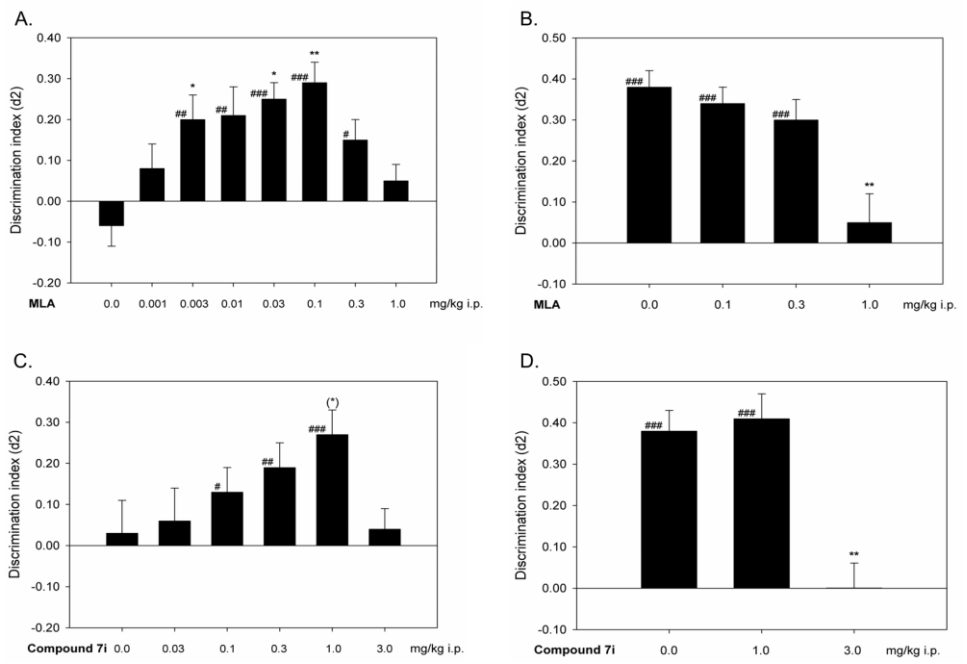


Figure 1. ORT acquisition studies with α7 nAChR antagonists. A) Effects of MLA (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). When compared to vehicle, MLA (0.003, 0.03 and 0.1 mg/kg administered 30 min before T1) prevented loss of long-term memory. **B)** Effects of MLA (i.p., administered 30 min before T1) in a 1 h retention interval ORT (mean ± SEM). When compared to vehicle, MLA (1.0 mg/kg) induced a memory deficit in this paradigm. **C)** Effects of Compound 7i (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). When compared to vehicle, Compound 7i (1.0 mg/kg administered 15 min before T1) showed a tendency to prevent loss of long-term memory. **D)** Effects of Compound 7i (i.p., administered 15 min before T1) in a 1 h retention interval ORT (mean ± SEM). When compared to vehicle, Compound 7i (3.0 mg/kg) induced a memory deficit in this paradigm. Differences from vehicle (ANOVA): (*)p = 0.1; *p < 0.05; **p < 0.01. Differences from chance performance (one-sample t-tests): $^{\#}p$ < 0.05; $^{\#\#}p$ < 0.01; $^{\#\#\#}p$ < 0.001. n = 17-18 per treatment.

ORT: memory consolidation processes: MLA

In the 24 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{2,34}$ = 2.71; P = 0.081). In T2, a difference was found for the exploration times between the different treatment conditions (e2, $F_{2,34}$ = 7.34; P = 0.002)**.** Post hoc analyses revealed significantly lower exploration times in T2 for the 0.1 mg/kg MLA condition when compared to both the vehicle (P = 0.009) and the 0.03 mg/kg MLA condition (P = 0.029). Furthermore, the d2 indices did not differ between treatment conditions ($F_{2,34}$ = 0.13; P = 0.876). Likewise, one-sample t-statistics showed that none of the discrimination performances were significantly different from chance level in any of the experimental conditions (Figure 2A).

ORT: memory consolidation processes: Compound 7i

In the 24 h retention interval, no differences were found between treatment conditions on the level of exploration in T1 (e1, $F_{2,34}$ = 3.11; P = 0.057) or T2 (e2, $F_{2,34}$ = 2.16; P = 0.131)**.** Furthermore, the d2 indices did not differ between treatment conditions ($F_{2,34}$ = 0.06; P = 0.947). Likewise, one-sample t-statistics showed that none of the discrimination performances was significantly different from chance level in any of the experimental conditions (Figure 2B).

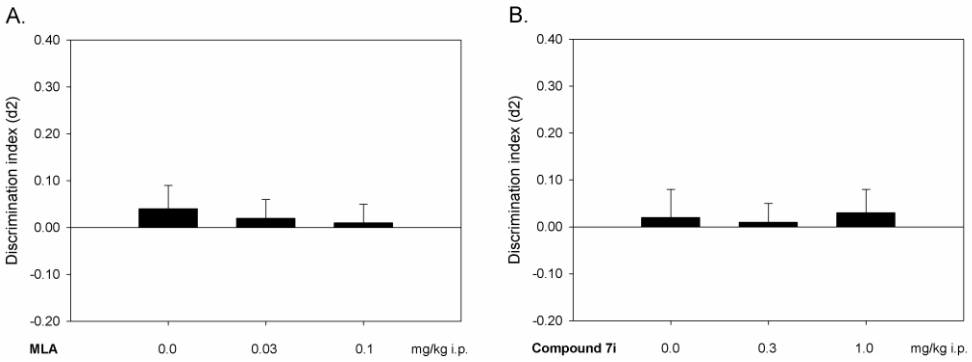


Figure 2. ORT consolidation studies with α7 nAChR antagonists. A) Effects of MLA (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). When compared to vehicle, optimum doses of MLA (0.03 and 0.1 mg/kg administered 4-10 min after T1) did not prevent loss of long-term memory. **B)** Effects of Compound 7i (i.p.) on the d2 index using a 24 h retention interval (mean ± SEM). When compared to vehicle, optimum doses of Compound 7i (0.3 and 1.0 mg/kg administered 4-10 min after T1) did not prevent loss of long-term memory. n = 18 per treatment.

Electrophysiology: MLA

Electrophysiological experiments were performed by using a weak tetanus to produce E-LTP, i.e. a transient potentiation at Schaffer collateral-CA1 synapses in hippocampal slices. Similar to the behavioral studies, a 10 min perfusion with a low concentration of MLA (100nM) before tetanus produced a longer lasting potentiation (225.66 ± 33.55 vs. 132.62 ± 4.30% of baseline slope, n = 7/7 slices; $F_{1,12}$ = 9.86, P = 0.009; Fig. 3A), whereas no changes in E-LTP were recorded when MLA was administered after tetanus (129.74 ± 13.43% of baseline slope, n = 6 slices; $F_{1,11}$ = 1.54, P = 0.240; Fig. 3A). A high concentration of MLA (10µM) administered before tetanus did not induce a longer-lasting potentiation (130.08 ± 2.81% of baseline slope, n = 6 slices; $F_{1,11}$ = 0.64, P = 0.44; Fig. 3B).

Electrophysiology: Compound 7i

Again, like the behavioral studies, a low concentration of Compound 7i (100nM) produced a longer lasting potentiation compared to vehicle when administered before (221.70 ± 12.84 vs. 139.55 ± 5.76% of baseline slope, n = 7/7 slices; $F_{1,12}$ = 18.43, P = 0.001; Fig. 3C), but not after a weak tetanus (155.03 ± 10.04% of baseline slope, n = 6 slices; $F_{1,11}$ = 2.801, P = 0.122; Fig. 3C). Perfusion of hippocampal slices with a high concentration of Compound 7i (1µM) did not modify potentiation (128.70 ± 12.46% of baseline slope, n = 6 slices; $F_{1,11}$ = 0.846, P = 0.377; Fig. 3D).

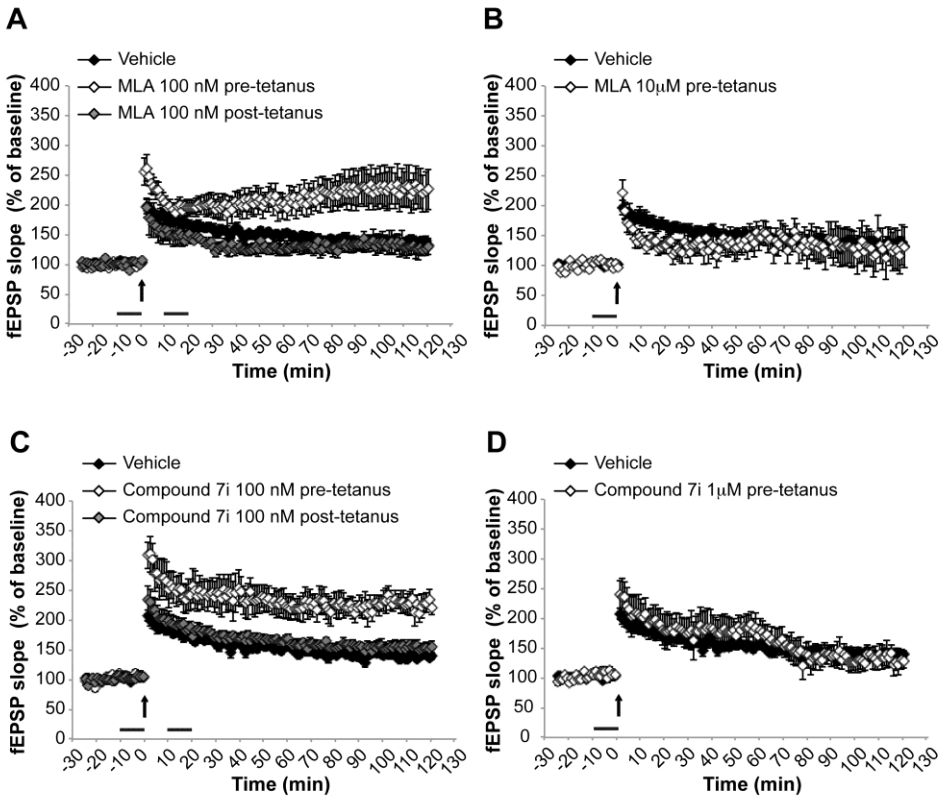


Figure 3. Effects of α7 nAChR antagonists on hippocampal LTP. A) Ten minutes perfusion of hippocampal slices with a low dose (100nM) before, but not after tetanus increased levels of potentiation when compared to vehicle treated slices. **B)** High dose administration of MLA (10μM) did not lead to any differences in the level of potentation. **C)** Likewise, treatment with a low dose (100nM) of Compound 7i before tetanus increased levels of potentiation, whereas no changes were recorded when Compound 7i was administered after tetanus. **D)** A high dose of Compound 7i (1μM) after LTP induction did not lead to any differences in the level of potentation. Data represent mean ± SEM. Arrow indicates tetanus delivery (one 10-burst stimulation – weak tetanus) and horizontal bars indicate the period during which drugs were added to the bath solution.

Microdialysis: MLA

Glutamate efflux was increased after 1.0 mg/kg MLA administration ($F_{16,49}$= 4.32; P = 0.000). These effects were seen from dialysate 9 onwards, extending for at least up to 2 h (fraction 17). Fraction 9 corresponds to 30 minutes after MLA administration, which resembles the injection/testing regimen of the ORT study.

ANOVA also showed an effect in de vehicle condition ($F_{16,45}$ = 2.15; P = 0.023). Post hoc analysis showed that the last dialysate was significantly lower (P = 0.042) when compared to the dialysate at fraction 5 (baseline). Because of this effect, independent samples *t*-tests were also performed to check whether 1.0 mg/kg MLA also differed from the vehicle/control condition per fraction. The glutamate efflux after 1.0 mg/kg MLA was significantly higher than the vehicle/control condition from fraction 10 (40 minutes after the injection) onwards (Figure 4A). For GABA, effects were found for both the 0.01 mg/kg MLA condition ($F_{16,30}$ = 2.45; P = 0.016) and for the vehicle/control condition ($F_{16,48}$ = 3.71; P = 0.000). Post hoc analysis revealed a slight but significant decrease of GABA at dialysate 12, 14 and 16 (compared to dialysate fraction 5), which correspond to 70, 90 and 110 min, respectively, after 0.01 mg/kg MLA administration (dialysate 15 and 17 showed a trend; P < 0.08). The vehicle condition showed a significant decrease at the last dialysate measurements (15-17 when compared to dialysate fraction 5). Independent samples *t*-tests showed that the GABA efflux after 0.01 mg/kg MLA was significantly lower than the vehicle/control condition per fraction at fraction 12, 14, 16 and 17 (Figure 4B).

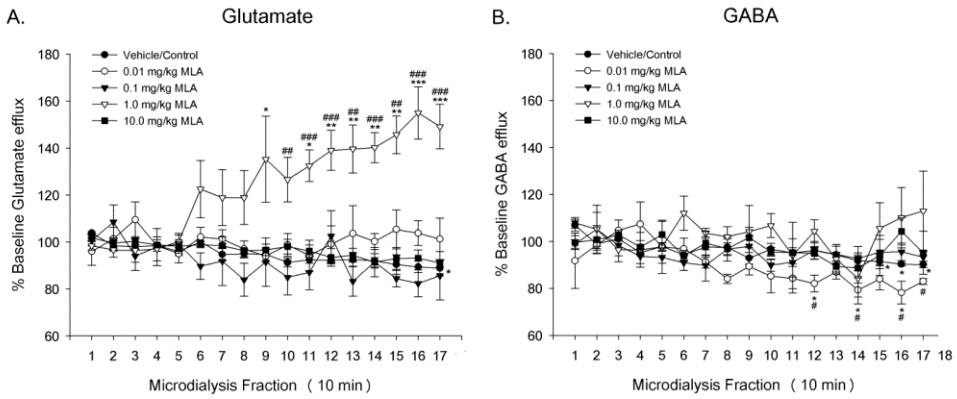


Figure 4. Amino acid efflux at baseline and after treatment with 0-10 mg/kg MLA (i.p.) at dialysate fraction 6. A) Glutamate efflux was significantly increased from 30 min onwards after 1.0 mg/kg MLA treatment (dialysate fraction 9) when compared to both baseline level (dialysate fraction 5) and to the vehicle/control condition per fraction point. **B)** In contrast, GABA efflux was significantly decreased around 60 min after 0.01 mg/kg MLA treatment (dialysate fraction 12) when compared to both baseline level (dialysate fraction 5) and to the vehicle/control condition per fraction. Data represent mean ± SEM. Differences from dialysate fraction 5 (baseline) (ANOVA): *p < 0.05; **p < 0.01; ***p < 0.001; Differences from vehicle/control group (independent samples *t*-tests): $^{\#}p$ < 0.05; $^{\#\#}p$ < 0.01; $^{\#\#\#}p$ < 0.001, n = 3-5 per treatment condition.

Discussion

The objective of this study was to investigate the effects of low dose administration of α7 nAChR antagonists on memory function in rodents. Memory performance was assessed with the ORT after administration of the α7 nAChR antagonists MLA or Compound 7i. Specifically, both memory acquisition and memory consolidation processes were assessed in this paradigm (by administrating the drug before or after T1, respectively). It was found that both MLA and Compound 7i improved the memory acquisition process of rats in the ORT natural forgetting (i.e. 24h retention interval) paradigm (optimal dose range: MLA; 0.003-0.1 mg/kg, i.p. and Compound 7i; 0.1-1.0 mg/kg, i.p.). Moreover, it was found that a dose that was too high to improve memory in the natural forgetting paradigm (MLA; 1.0 mg/kg, i.p. and Compound 7i; 3.0 mg/kg, i.p.), was also sufficient to induce a memory deficit in the 1 h retention interval ORT, an interval that normally leads to good memory performance of rats (e.g. Prickaerts et al., 2012). In addition, we showed that the optimum doses of both MLA and Compound 7i did not improve the memory consolidation processes. The optimum doses of both compounds were determined in the ORT memory acquisition process experiments. Electrophysiological studies showed that low concentrations MLA or Compound 7i induced a longer-lasting potentiation when administered before a weak tetanus. Conversely, perfusion of hippocampal slices with low concentrations of α7 nAChR antagonists after LTP induction or high concentrations before tetanus did not modify potentiation. Microdialysis studies showed that 1.0 mg/kg MLA led to a significant increase of glutamate efflux 30 minutes after administration that lasted up to at least 2 h after administration. Furthermore, 0.01 mg/kg MLA led to a slight decrease of GABA efflux 60 minutes after MLA administration.

In order to investigate the effects of the α7 nAChR antagonists on the memory acquisition processes, MLA was i.p. administered 30 min before T1. This administration time was chosen because previous research on MLA indicated a half-life of 37 ± 4 min and a peak brain concentration at 30 min after i.p. administration in rats (Turek et al., 1995). The administration time of Compound 7i (15 min before T1), was chosen because of the peak concentration in both plasma and brain after 15 min following i.p. administration in mice (Peng et al., 2010). This was in accordance with our objective to target the memory acquisition process, although effects on memory consolidation processes could not be ruled out by only these experiments (van Goethem et al., 2012). Therefore the additional ORT experiments to investigate the effects of the α7 nAChR antagonists specifically on memory consolidation processes were performed as well. In these experiments, both compounds were administered

i.p. after T1 (Bollen et al., 2014). The results showed that both MLA and Compound 7i had no effect on memory consolidation processes. This is in line with the electrophysiological studies, where it was shown that a low dose of α7 nAChR antagonists before, but not after LTP induction led to a longer-lasting potentiation. Therefore, both these behavioral and electrophysiological studies suggest that α7 nAChR antagonism only has effects on the acquisition-like memory process.

In the ORT memory consolidation study, an effect of MLA was found on the exploratory behavior of the rats in T2. Specifically, the exploration behavior of the rats in the 0.1 mg/kg condition was lower than in the vehicle or 0.03 mg/kg MLA condition. Since T2 was almost 24 h after the MLA injection, the found difference in exploratory behavior was unlikely to be related to the drug condition. Specifically, the differences between drug conditions were not stable over T1 and T2, something that would be expected considering the short half-life (± 37 min) and fast peak brain concentration (30 min) of MLA in rats (Turek et al., 1995). Despite the statistical significant differences in exploratory behavior in T2, the mean exploration times of the animals was always sufficient (>20 sec, data not shown) to draw reliable conclusions (Akkerman et al., 2012). Sporadic changes in exploratory behavior can occur during behavioral testing, and often the exact reasons remain unknown. Most importantly, no impairment in locomotor activity was found according to the exploratory behavior in the ORT (i.e. sufficient exploration times). Therefore, we interpret this difference in the amount of exploratory behavior to be incidental.

MLA also interacts with α6, α9, α9α10, α4β2 and α6β2 nAChRs at, at least 30 fold lower affinity than on α7 nAChRs (Hahn et al., 2011). Because of the lower affinity for these receptors, it seems unlikely that the procognitive effects observed after such low dose MLA administration were due to interaction with these receptors. Compound 7i shows high selectivity for the α7 nAChRs (Peng et al., 2010).

In a recent study measuring hippocampal neurotransmitter efflux in rats, in vivo microdialysis was paired with an ORT paradigm. It was found that during object exposure in both trials, GABA efflux was unaffected. However, while glutamate efflux did not increase above baseline levels during exposure of familiar objects, it was significantly increased when a novel object was shown 2 h later in the second ORT trial. This strongly suggests that hippocampal glutamate is involved in memory formation processes (Stanley et al., 2012). The results found in that study might explain the discrepancy in active MLA doses of our ORT (0.1 mg/kg)

and microdialysis studies (1 mg/kg). To elaborate on this, considering the increased glutamate efflux during exposure to novel objects alone, the combination of MLA administration plus novel object exposure would probably lead to additive glutamate effects. This could explain the difference in active doses. We hypothesize that combining microdialysis with an ORT paradigm could have led to lower active MLA doses, i.e. that glutamate efflux is more optimal with lower MLA doses when combined with novelty exposure. Thus, an increase in glutamate, when a novel object is shown to rats, could in addition to an increase in glutamate mediated via the 1 mg/kg MLA administration, possibly lead to a 'glutamate overstimulation' (due to additive/accumulation effects of glutamate). This in turn might cause the cognitive impairing effect of 1 mg/kg MLA seen in our ORT studies (Figure 1A). Following this rationale, a lower dose of MLA, could in combination with a novel object already optimally increase glutamate release and thus improve memory formation processes. This however remains to be investigated in future studies.

Hypothetical mechanisms of action at low doses of α7 nAChR antagonists could be alterations in the rate of desensitization or re-sensitization of these receptor ion channels. The agonist binding domain of the nAChR is at the interface between the α-subunit and the neighboring subunit (Arias, 2000), leading to as many as five functional agonist binding sites for the homopentameric α7 nAChR. nAChRs generally require occupancy of two agonist binding sites (by ACh or agonist) to achieve channel opening (Cachelin and Rust, 1994), leaving in the case of α7 nAChRs three additional sites on this homopentameric receptor available to bind antagonist. It is plausible that from these binding sites, the desensitization of the receptor is modulated. Conversely, low doses of α7 nAChR antagonists may promote α7 nAChR re-sensitization. By occupying a subset of α7 nAChRs, these receptors have an opportunity to 'recover' or re-sensitize. Subsequently, after antagonist binding, a full response to agonists (like e.g. ACh) can be produced after the receptors have been re-sensitized. In practice, this would mean that low doses of antagonists somehow transform the conformational state of the ion channel into a state which is more susceptible to agonist binding. Yet other suggested mechanisms are that α7 nAChR desensitization (Fujii et al., 2000; Wang and Sun, 2005; Hahn et al., 2011) or a net decrease of receptor activity (Burke et al., 2014) has beneficial effects on cognitive performance. By administrating an α7 nAChR antagonist, the effects of α7 nAChR desensitization are mimicked and hence the observed cognitive improvement could be linked to receptor desensitization.

In addition, it cannot be ruled out is that these antagonists function as modulators, which would mean that they have different binding sites when compared to ACh or other agonists. In this explanation, the receptor recycling is somehow modulated by this mechanism, thereby preventing desensitization of the system. This would mean that the used α7 nAChR antagonists may somehow modulate desensitization from a distinct binding site. Although possible, we regard this hypothesis rather unlikely, considering it has been established that both MLA and Compound 7i are competitive antagonists and, as such, occupy the same binding site as ACh (Palma et al., 1996; Dani, 2001; Peng et al., 2010).

Finally, an alternative explanation for these phenomena could be that low doses of α7 nAChR antagonists act as mild agonists, while having antagonistic properties in higher doses (mixed agonist-antagonist activity; e.g. Pick et al., 1997). Similarly, this double role exerted by α7 nAChRs antagonists might be related to the biphasic dose-response phenomenon of hormesis, characterized by low-dose stimulation and high-dose inhibition (Calabrese, 2008). Indeed, while high doses of a stressor can damage a biological system, the same substance, at low doses, can positively stimulate several physiologic functions, including cognition (Puzzo et al., 2012). While there are, to our knowledge no published results that indicate that MLA has a mixed agonist-antagonist activity, this effect cannot be ruled out, and remains to be investigated in future studies. Although, given the fact that the two (chemically diverse) α7 nAChR antagonists showed the same profile in behavioral and electrophysiological studies, the chances of both compounds having such a mixed/double activity, are considered small. Unless such a biological response is a hallmark response α7 nAChRs have to their antagonists. This however, also remains speculative.

In summary, blocking α7 nAChRs with low doses of selective antagonists improves specifically the memory acquisition process, but not the memory consolidation processes. Moreover, while the main focus of the α7 nAChR as a target for cognition enhancement has traditionally involved agonists and positive modulators, antagonists at appropriate doses may also prove to be a valuable tool for cognition enhancement in for instance AD or schizophrenia.

References

Akkerman, S., Blokland, A., Reneerkens, O., van Goethem, N. P., Bollen, E., Gijselaers, H. J. M., et al. (2012). Object recognition testing: Methodological considerations on exploration and discrimination measures. *Behavioural Brain Research, 232*(2), 335-347.

Arias, H. R. (2000). Localization of agonists and competitive antagonist binding sites on nicotinic acetylcholine receptors. *Neurochemistry International, 36*(7), 595-645.

Arias, H. R., Feuerbach, D., Bhumireddy, P., Ortells, M. O. (2010). Inhibitory mechanisms and binding site location for serotonin selective reuptake inhibitors on nicotinic acetylcholine receptors. *The International Journal of Biochemistry & Cell biology, 42*(5), 712-724.

Bliss, T. V., and Collingridge, G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature 361*, 31-39.

Bollen, E., Puzzo, D., Rutten, K., Privitera, L., De Vry, J., Vanmierlo, T., et al. (2014). Improved Long-Term Memory via Enhancing cGMP-PKG Signaling Requires cAMP-PKA Signaling. *Neuropsychopharmacology, 39*, 2497-2505.

Burke, D. A., Heshmati, P., Kholdebarin, E., Levin, E. D. (2014). Decreasing nicotinic receptor activity and the spatial learning impairment caused by the NMDA glutamate antagonist dizocilpine in rats. *European Journal of Pharmacology, 741*, 132-139.

Cachelin, A. B., and Rust, G. (1994). Unusual pharmacology of (+)-tubocurarine with rat neuronal nicotinic acetylcholine receptors containing beta 4 subunits. *Molecular Pharmacology, 46*(6), 1168-1174.

Calabrese, E.J. (2008). Hormesis and medicine. *British Journal of Clinical Pharmacology, 66*(5), 594-617.

Cavallero, A., Marte, A., Fedele, E. (2009). L-Aspartate as an amino acid neurotransmitter: mechanisms of the depolarization-induced release from cerebrocortical synaptosomes. *Journal of Neurochemistry, 110*(3), 924-934.

Chapman, P. F., White, G. L., Jones, M. W., Cooper-Blacketer, D., Marshall, V. J., Irizarry, M., et al. (1999). Impaired synaptic plasticity and learning in aged amyloid precursor protein transgenic mice. *Nature Neuroscience, 2*, 271-276.

Dani, J. A. (2001). Overview of nicotinic receptors and their roles in the central nervous system. *Biological Psychiatry, 49*(3), 166-174.

Dani, J. A., and Bertrand, D. (2006). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Review of Pharmacology and Toxicology, 47*, 699-729.

Dickinson, J. A., Kew, J. N., Wonnacott, S. (2008). Presynaptic α7-and β2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. *Molecular Pharmacology, 74*(2), 348-359.

Ennaceur, A., and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research, 31*(1), 47-59.

Fujii, S., Ji, Z., & Sumikawa, K. (2000). Inactivation of α7 ACh receptors and activation of non-α7 ACh receptors both contribute to long term potentiation induction in the hippocampal CA1 region. *Neuroscience Letters, 286*(2), 134-138.

Hahn, B., Shoaib, M., Stolerman, I. P. (2011). Selective nicotinic receptor antagonists: effects on attention and nicotine-induced attentional enhancement. *Psychopharmacology, 217*(1), 75-82.

Kandel, E. R. (2012). The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Molecular Brain, 5*(14), 1-12.

Levin, E. D., and Simon, B. B. (1998). Nicotinic acetylcholine involvement in cognitive function in animals. *Psychopharmacology, 138*(3), 217-230.

Mansvelder, H. D., and McGehee, D. S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron, 27*(2), 349-357.

Molas, S., and Dierssen, M. (2014). The role of nicotinic receptors in shaping and functioning of the glutamatergic system: A window into cognitive pathology. *Neuroscience & Biobehavioral Reviews, 46*(2), 315-325.

Newhouse, P., Potter, A., Levin, E. (1997). Nicotinic system involvement in Alzheimer's and Parkinson's diseases. Implications for therapeutics. *Drugs & Aging, 11*(3), 206-228.

Newhouse, P. A., Potter, A., Singh, A. (2004). Effects of nicotinic stimulation on cognitive performance. *Current Opinion in Pharmacology, 4*(1), 36-46.

Palma, E., Bertrand, S., Binzoni, T., Bertrand, D. (1996). Neuronal nicotinic α7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *Journal of Physiology, 491*(1), 151-161.

Paxinos, G. and Watson, C. (1986). The rat brain in stereotaxic coordinates. *Academic Press*, San Diego.

Peng, Y., Zhang, Q., Snyder, G. L., Zhu, H., Yao, W., Tomesch, J., et al. (2010). Discovery of novel α7 nicotinic receptor antagonists. *Bioorganic & Medicinal Chemistry Letters, 20*(16), 4825-4830.

Picciotto, M. R., Caldarone, B. J., King, S. L., Zachariou, V. (2000). Nicotinic receptors in the brain: links between molecular biology and behavior. *Neuropsychopharmacology, 22*, 451-465.

Pick, C. G., Peter, Y., Schreiber, S., Weizman, R. (1997). Pharmacological characterization of buprenorphine, a mixed agonist–antagonist with κ3 analgesia. *Brain Research, 744*(1), 41-46.

Prickaerts, J., van Goethem, N. P., Chesworth, R., Shapiro, G., Boess, F. G., Methfessel, C., et al. (2012). EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacology, 62*(2), 1099-1110.

Puzzo, D., Privitera, L., Palmeri, A. (2012). Hormetic effect of amyloid-beta peptide in synaptic plasticity and memory. *Neurobiology of Aging, 33*(7), 1484.e15-1484.e24.

Puzzo, D., Staniszewski, A., Deng, S. X., Privitera, L., Leznik, E., Liu, S., et al. (2009). Phosphodiesterase inhibition improves synaptic function, memory, and amyloid-beta load in an Alzheimer's disease mouse model. *Journal of Neuroscience, 29*(25), 8075-8086.

Quarta, D., Naylor, C. G., Barik, J., Fernandes, C., Wonnacott, S., Stolerman, I. P. (2009). Drug discrimination and neurochemical studies in α7 null mutant mice: tests for the role of nicotinic α7 receptors in dopamine release. *Psychopharmacology, 203*(2), 399-410.

Ricciarelli, R., Puzzo, D., Bruno, O., Canepa, E., Gardella, E., Rivera, D., et al. (2014). A novel mechanism for cyclic adenosine monophosphate-mediated memory formation: Role of amyloid beta. *Annals of Neurology, 75*(4), 602-607.

Stanley, E. M., Wilson, M. A., Fadel, J. R. (2012). Hippocampal neurotransmitter efflux during one-trial novel object recognition in rats. *Neuroscience Letters, 511*(1), 38-42.

Toyohara, J., and Hashimoto, K. (2010). α7 nicotinic receptor agonists: potential therapeutic drugs for treatment of cognitive impairments in schizophrenia and Alzheimer's disease. *The Open Medicinal Chemistry Journal, 4*, 37-56.

Turek, J. W., Kang, C. H., Campbell, J. E., Arneric, S. P., Sullivan, J. P. (1995). A sensitive technique for the detection of the α7 neuronal nicotinic acetylcholine receptor antagonist, methyllycaconitine, in rat plasma and brain. *Journal of Neuroscience Methods, 61*(1), 113-118.

van Goethem, N. P., Rutten, K., van der Staay, F. J., Jans, L. A., Akkerman, S., Steinbusch, H. W., et al. (2012). Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research, 232*(2), 323-334.

Wang, H., and Sun, X. (2005). Desensitized nicotinic receptors in brain. *Brain Research Reviews, 48*(3), 420-437.

Ward, J. M., Cockcroft, V. B., Lunt, G. G., Smillie, F. S., Wonnacott, S. (1990). Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites. *FEBS letters, 270*(1-2), 45-48.

Zakharenko, S. S., Patterson, S. L., Dragatsis, I., Zeitlin, S. O., Siegelbaum, S. A., Kandel, E. R., et al. (2003). Presynaptic BDNF required for a presynaptic but not postsynaptic component of LTP at hippocampal CA1-CA3 synapses. *Neuron, 39*(6), 975-990.

Chapter 9

General Discussion and Conclusions

Aim of this dissertation

The aim of this dissertation was to investigate the role of α7 nicotinic acetylcholine receptors (α7 nAChRs) in memory processes. α7 nAChR functioning was assessed on a behavioral level with the object recognition task (ORT) or its spatial variant, the object location task (OLT). Furthermore, α7 nAChR functioning was assessed on a mechanical level with electrophysiological recordings *in vitro*. In addition, the specific characteristics of a novel α7 nAChR ligand (EVP-6124) were determined in binding assays and pharmacokinetic studies. The goal was to evaluate different possibilities to utilize the α7 nAChR for treatment possibilities in different psychiatric and neurological disorders. The focus was on Alzheimer's disease (AD) and schizophrenia.

Chapter 2, 3 and 4 gave a complete overview of the memory processes which were studied and the methodologies that were used to study them. In chapter 2, a comprehensive model of recognition and recall was given which synthesized seemingly contradictory processes and findings from the literature. This chapter explained how in the ORT and OLT, object memory and spatial memory are respectively being utilized. As indicated in chapter 3, it should be noted that that ORT/OLT outcomes do not have to solely reflect memory processes. Other cognitive processes needed for memory formation (e.g. attention processes), probably also contribute to proper memory formation. Indeed, the involvement of acetylcholine (ACh) and cholinergic drugs in attention processes instead of specific memory processes has been put forward earlier (Blokland, 1995). This

shows that, in behavioral testing, it is rather difficult to see these cognitive processes as separate since cholinergic drugs enhance ORT performance (e.g. Prickaerts et al., 2005).

Chapter 3 described the ORT and the possible pitfalls accompanying this task. At present the ORT has a strong position as a primary behavioral screen for testing putative memory enhancing drugs. The paradigm is well-suited to assess memory acquisition, consolidation and retrieval processes, while avoiding reinforcement or rule learning. When keeping the in chapter 3 mentioned considerations in mind, the reliability of the results is warranted and the ORT continues to be a valuable tool in behavioral and pharmacological research.

Chapter 4 focused on how memory deficits as encountered in different psychiatric and neurological disorders can be modelled in rodents. Pharmacological deficit, aging as well as transgenic rodent models were described. Furthermore the face, construct and predictive validities of these different models were discussed. The goal of animal research is to mimic as much as possible the human disease pathophysiology, and thus improving construct and face validity will provide greater insights into basic genetic and molecular mechanisms involved in the expression of behavior (D'Mello and Steckler, 1996). At the same time, once these mechanisms are better understood, predictive validity will improve and better efficacious therapeutic strategies can be explored and developed for finding treatments for cognitive deficits in human patients. Chapter 4 also provided valuable background information for chapter 7, in which a pharmacological deficit model for schizophrenia was further validated.

In this dissertation, three rodent models have been used to test the cognition enhancing properties of different α7 nAChR ligands in the ORT and OLT. These models were: a natural forgetting (or time-dependent forgetting) model, a scopolamine-induced deficit model and a subchronic MK-801-induced deficit model. A natural forgetting paradigm is often used to test the 'clean' effect of a drug, i.e. to investigate if a drug can enhance the memory performance of naïve rodents not exposed to a deficit model. In this model, a longer time-delay between ORT trials is used (i.e. 24 h). The scopolamine- and MK-801-induced deficit models are mostly utilized to model AD and schizophrenia, respectively. Both drugs alter distinct neurotransmitter systems which are dysfunctional in the respective disorders. As such, scopolamine is a cholinergic (non-selective muscarinic M1-M5) receptor antagonist and MK-801 is a glutamatergic (NMDA) receptor antagonist. Both AD and schizophrenia have been linked to α7 nAChR

functioning (e.g. Toyohara and Hashimoto, 2010). In chapter 5-8 we further investigated the potential therapeutic value of different α7 nAChR ligands in these disorders. Several studies were performed in order to further increase our understanding of the role these receptors, and their different ligands, play in enhancing different memory processes. The following paragraphs provide more background information while discussing the results of our studies with α7 nAChR partial and full agonists and antagonists.

α7 nAChRs and transient effects

Cognition

The identification of high expression levels of neuronal nAChRs in many brain areas, including the hippocampus and cortex, and more specifically of those containing homopentameric α7-subunits, suggested that these receptors may play an important role in cognitive functions (Dani and Bertrand, 2007). This hypothesis was confirmed by the finding that specific agonists of α7 nAChRs improved memory performance in animals, as shown initially by the effects of molecules such as GTS-21 (reviewed in: Kem, 2000) and more recently by more selective and potent agonists (e.g. reviewed in: Wallace and Porter, 2011; Posadas et al., 2013).

In humans, different α7 nAChR agonists and modulators have been investigated for their potential to improve memory and attention disorders encountered in for instance AD and schizophrenia (Toyohara and Hashimoto, 2010). Phase I and II clinical trials showed that α7 nAChR ligands had beneficial effects on the cognitive function of humans. These improvements related to (episodic) memory as well as working memory and attention (Toyohara and Hashimoto, 2010; Preskorn et al., 2014). A major drawback of α7 nAChRs is that they show rapid desensitization following exposure to agonists (Picciotto et al., 2000). This desensitization could translate into a quick build-up for tolerance to agonists designed to activate α7 nAChRs. Possible causes of desensitization remain elusive. But considering the permeability of α7 nAChRs to Ca^{2+}, avoiding excitotoxicity might be a function of the desensitization process. Furthermore, since nicotine itself shows low affinity for α7 nAChRs, selective α7 nAChR agonists are not considered to have addictive properties (Dani and Bertrand, 2007).

α7 nAChRs and tonic effects

Neuroprotection

Agonists and partial agonists of nAChRs have been demonstrated to have neuroprotective effects in the CNS. For example, it has been shown that nicotine delays the aging process of nigrostriatal neurons (Prasad et al., 1994). Furthermore, nicotine has been demonstrated to have protective effects against exocytotic cell death (Marin et al., 1994). These protective effects have been linked to various mechanisms, including; increased expression of neurotrophic factors (Galzi et al., 1996), activated protein kinase C (Li et al., 1999) and inhibition of nitric oxide production (Shimohama et al., 1996). These neuroprotective effects have been correlated with activation of α7 nAChRs (Jonnala and Buccafusco, 2001). The weak partial agonist GTS-21 has been shown to exhibit neuroprotective effects against glutamate-induced and ischemic neurotoxicity (Nanri et al., 1998; Shimohama et al., 1998).

It has been hypothesized that Ca^{2+} entry through α7 nAChRs can promote the survival of neurons (Dajas-Bailador et al., 2000). However, Ca^{2+} entry is also known to be neurotoxic. The degree of Ca^{2+} influx is probably a very important factor when determining if an outcome will be neuroprotective or neurotoxic. Like already explained in the introduction chapter of this dissertation (Chapter 1), a partial agonist can act both as an agonist and as an antagonist depending on the surrounding concentration of endogenous neurotransmitter. In short, in the case of high endogenous neurotransmitter, the partial agonist will have an antagonistic effect; as such it will reduce the maximal response of the endogenous neurotransmitter. When endogenous neurotransmitter levels are low, the partial agonist will have an agonist effect and activate receptors. Which of these properties of a partial agonist is most important for its activity is often difficult, if not impossible, to determine. Precise measurements of the actual concentrations of the endogenous neurotransmitters in the brain at a given moment are needed in order to draw reliable conclusions about this. Following this rationale, the neuroprotective effect of partial agonists might be caused by their lower efficacy, suggesting that weaker receptor stimulation may favor neuroprotection, e.g. more favorable Ca^{2+} influx (Hogg and Bertrand, 2007). In addition, from what we have learned in chapter 8, low dose antagonist exposure might function analogously, by causing fewer and shorter channel openings when compared full agonists.

Functional upregulation

In 1983, Marks et al. showed an upregulation of the number of ACh binding sites in the brains of mice after chronic exposure to nicotine. Later, it was found that also in the brains of smokers, a significant upregulation of high affinity binding sites for nicotine was present (Wonnacott, 1990). This showed the ability of nAChR ligands to increase the density of ACh binding sites in the CNS (Hogg and Bertrand, 2007). Subsequently, it was discovered that different full and partial agonists of nAChRs also produced an upregulation of ACh binding sites. In addition, it has been reported that nAChR antagonists caused an upregulation of α4β2 nAChRs *in vitro* (Peng et al., 1994; Gopalakrishnan et al., 1997). From these last studies it was concluded that activation of receptors is not required for upregulation. Furthermore, it seems that this upregulation is also not related to the efficacy of a ligand to activate a receptor (Hogg and Bertrand, 2007).

Likewise, different α7 nAChR partial agonists (e.g. epibatidine, GTS-21, DMPP) caused an upregulation of α7 nAChR binding sites *in vitro*. Furthermore, the α7 nAChR antagonist MLA showed the same *in vitro* upregulation of α7 nAChR binding sites (Molinari et al., 1998).

It is not exactly clear what this change in high affinity binding sites reflects. On the one hand, it might be that the number of receptors is increased. On the other hand, however, it might also be that a change in the affinity of the already existing receptors is induced (Hogg and Bertrand, 2007). In any case, this increase in ligand binding has been associated with enhanced receptor functioning. The proper terminology hereof is functional upregulation (Rowell and Wonnacott, 1990). Functional upregulation could be beneficial in chronic treatment of disorders in which nAChR function is dysfunctional (Hogg and Bertrand, 2007). Whether this is the case for specifically α7 nAChRs remains to be investigated (given the rapid desensitization of these receptors following exposure to agonists (Picciotto et al., 2000)).

α7 nAChRs and disorders

Alzheimer's disease

AD is a neurodegenerative disorder which affects almost 10% of individuals over the age of 65 and is behaviorally characterized by cognitive dysfunction, mainly related to memory processes (Oddo and LaFerla, 2006). Loss of cholinergic neurons in the basal forebrain, as well as progressive degeneration of the cholinergic innervation of the hippocampus and cerebral cortex, has been shown in AD patients (Delacourte and Defossez, 1986; Cummings and Benson,

1987). Furthermore, these patients show a reduction in the activity of the ACh synthesizing enzyme choline acetyl transferase in these regions (Coyle et al., 1983). These findings probably all contribute to dysfunctional memory in AD patients and have led to investigations into the possibilities to utilize cholinergic receptors for cognition enhancement (Toyohara and Hashimoto, 2010).

To compensate for the loss of cholinergic neurotransmission, acetylcholinesterase inhibitors (AChEIs) like donepezil are the current mainstay symptomatic treatments for AD patients. AChEIs inhibit the cleavage of ACh and thereby increase the level of this neurotransmitter in the synaptic cleft. The AChEIs can produce modest improvements in cognition, but are not free of side effects. These side effects include nausea, vomiting, diarrhea, abdominal cramping and anorexia and are the result of inhibition of acetylcholinesterase (AChE) in the periphery. The modest efficacy combined with this side effect profile tends to limit the clinical usefulness of these drugs (Lanctot et al., 2009).

Binding studies, which made use of labelled ligands, have indicated a loss of the α4β2 nAChRs in the brain of AD patients (Warpman and Nordberg, 1995; Hogg and Bertrand, 2007). In contrast, the results of most studies have shown no significant (Martin-Ruiz et al., 1999), or only minor loss (Burghaus et al., 2000) of α7-subunit protein levels in the temporal cortex of AD patients (Toyohara and Hashimoto, 2010). Another study has shown an upregulation of α7 nAChRs in cholinergic basal forebrain neurons in AD patients (Counts et al., 2007). It has been suggested that the upregulation of α7 nAChRs may occur as a compensatory mechanism to maintain neuronal function during AD progression (Ikonomovic et al., 2009; Toyohara and Hashimoto, 2010).

Another link between AD and α7 nAChRs form the β-amyloid (Aβ) peptides (1-42) and (1-40). Aβ peptides are found in high concentrations in neuritic plaques, one of the major hallmarks in AD pathology. It has been reported that Aβ peptides have their neurotoxic effects via α7 nAChRs (Forloni, 1996; Liu et al., 2001; Hogg and Bertrand, 2007). Aβ peptides bind with high affinity to α7 nAChRs (Wang et al., 2000a, 2000b). This interaction leads to intraneuronal accumulation of $A\beta_{42}$-α7 nAChR complexes (Nagele et al., 2002). It has been hypothesized that amyloid plaques in AD brains are actually the lysis remnants of degenerated, $A\beta_{42}$-overburdened neurons. Accordingly, the most vulnerable neurons appear to be those that abundantly express the α7 nAChR (D'Andrea and Nagele, 2006).

Other studies have shown that the interaction of Aβ peptides with α7 nAChRs leads to rapid tau phosphorylation (Wang et al., 2003), severe impairment of α7 nAChR channels (Liu et al., 2001) and neuronal cell death (Wang et al., 2000a). Moreover, it has been found that the number of α7 nAChRs on astrocytes is increased in the brain of AD patients (Lilja et al., 2011; Marutle et al., 2013). Microglia are activated by $A\beta_{42}$ and are involved in the clearance and phagocytosis of Aβ (Lee and Landreth, 2010). However, when too much reactive oxygen species are produced in this process, the neuroprotective function of microglia is reduced. Interestingly, stimulation of microglial α7 nAChRs inhibits the production of reactive oxygen species and this may favor the neuroprotective function again (e.g. Moon et al., 2008). This suggests that Aβ can have complicated functional interactions with α7 nAChRs both on neurons and glial cells. These findings indicate that chronic interaction of $A\beta_{42}$ with α7 nAChRs in the AD brain could cause cellular dysfunction and even neurodegeneration (Toyohara and Hashimoto, 2010).

Taken together, these findings suggest that α7 nAChRs are likely to play a role in Aβ-mediated AD pathology. This would suggest that α7 nAChR ligands may prove beneficial for therapeutic drugs for AD patients (Toyohara and Hashimoto, 2010). Hypothetically, this could mean a double mechanistic approach might get utilized here, i.e. symptomatic improvement via stimulation of hippocampal neuronal α7 nAChRs as well as pathology reduction by means of stimulation of microglial α7 nAChRs and disruption of the $A\beta_{42}$-α7 nAChR interaction in neurons.

Schizophrenia

Schizophrenia is a chronic disorder with a lifetime prevalence of nearly 1%. Causal factors of schizophrenia include complex genetic and environmental interactions. Schizophrenia is characterized by multiple symptoms, including; positive (i.e. hallucinations and delusions), negative (e.g. flattened affect, depression) and cognitive symptoms (CIAS, e.g. memory and attention problems) (Miyamoto et al. 2012).

Mainstay treatment of schizophrenia has focused on ameliorating positive symptoms by administration of dopamine type 2 (D_2) receptor antagonists, in line with the dopamine hypothesis of schizophrenia (Carlsson, 1988). However, many treated schizophrenic patients still have cognitive and negative symptoms of which the cognitive symptoms in particular place a burden on the patient's quality of life. By improving cognitive symptoms, the negative symptoms (and

hence functional outcome) of these patients could improve as well. Cognitive symptoms in schizophrenia might precede negative symptoms. In other words, the negative symptoms in schizophrenia might actually be the result of deficits in cognition, but this remains speculative. Some literature suggests that negative symptoms and cognitive deficits in schizophrenia share a common underlying substrate. This means that certain negative symptomes are relatively dependent on the cognitive deficits associated with this disorder (Rossi et al. 1997).

Currently, the focus of additional neurotransmitter systems which might play a role in schizophrenia has expanded beyond dopamine dysfunction, and now include neurotransmitter systems with nicotine/ACh, serotonin (5-HT) or glutamate (Miyamoto et al. 2012).

The involvement of nAChRs in schizophrenia was first suggested by the observation that a high percentage of schizophrenic patients are smokers (Lohr and Flynn, 1992). This gave rise to the hypothesis that nicotine intake might be a form of self-medication in order to compensate for a deficit in nicotinic neurotransmission (Goff et al., 1992; Hogg and Bertrand, 2007). Binding studies on post-mortem brain tissue from schizophrenic patients showed that binding to α7 nAChRs is reduced in the CA3 region of the hippocampus when compared to age-matched non-schizophrenic subjects (Freedman et al., 1995), suggestive of reduced nicotinic function. This is in contrast to non-schizophrenic smokers in whom high-affinity nicotinic binding sites were increased when compared to non-smokers (Wonnacott, 1990). Although this remains a rather controversial theory, it is possible that nicotine in cigarette smoke increases central nicotinic neurotransmission via the activation of nAChRs. This, in turn, might be beneficial for the cognitive function in schizophrenic patients (Hogg and Bertrand, 2007).

Preclinical and clinical studies have shown that α7 nAChR ligands were able to normalize the auditory gating deficit in both rodent models and in schizophrenic patients (Hogg and Bertrand, 2007; Toyohara and Hashimoto, 2010). This, together with the beneficial effects of α7 nAChR ligands on cognition, suggests that α7 nAChR ligands might prove beneficial as add-on therapy in the treatment of schizophrenic patients.

α7 nAChR partial agonism

EVP-6124 and memory processes

As reported in chapter 5, binding assays of EVP-6124 showed that this compound is selective for α7 nAChRs. Furthermore, functional electrophysiological assessment of EVP-6124 revealed that the compound acted as a partial agonist at α7 nAChRs. The memory enhancing effects of EVP-6124 *in vivo* were demonstrated in the ORT in a test of short-term memory impairment caused by scopolamine and in a test of natural forgetting, with a 24 h retention interval between the two trials. These data clearly illustrated that EVP-6124 improved memory in a dose-dependent manner (optimum dose: 0.3 mg/kg, p.o.), reaching a peak of activity at brain concentrations in the low nanomolar range.

To indicate that the observed pro-cognitive effect of EVP-6124 was specifically mediated via brain α7 nAChRs, an additional ORT study was performed in which the pro-cognitive effect of 0.3 mg/kg EVP-6124 in the natural forgetting paradigm was blocked by co-administration with the selective α7 nAChR antagonist methyllycaconitine (MLA) (0.3 mg/kg, i.p. or 10 µg, i.c.v.).

The performed binding assays also showed profound antagonist activity of EVP-6124 at 5-HT_3 receptors. Like stated in chapter 1, 5-HT_3 receptors and α7 nAChRs belong to the same super family of ligand-gated ion channels, and as such are homologues of each other. This 5-HT_3 antagonist activity seems to be a common feature of some of the recently characterized compounds, such as AZD0328 and RG3487 (Sydserff et al., 2009; Wallace et al., 2011), and could prove beneficial in reducing the potential emetic effects of nicotinic agonists, since 5-HT_3 receptor antagonists have been used for the treatment of nausea (Hashimoto, 2009). It has also been postulated that cognition might actually be enhanced via this 5-HT_3 antagonism (for a review, see: Walstab et al., 2010). However, the selective 5-HT_3 receptor antagonist ondansetron (0.1-10 mg/kg, p.o.) did not prevent natural forgetting in an ORT, while the α7 nAChR agonist RG3487 was effective (Wallace et al., 2011). Additionally, the α7 nAChR agonist AZD0328, which also shows a high affinity for 5-HT_3 receptors, was not effective in an ORT in mice lacking α7 nAChRs (Sydserff et al., 2009). Furthermore, we assume that the brain concentrations of EVP-6124 and other α7 nAChR agonists that are required for efficacy in cognitive tests (sub- to low nanomolar) are below the concentrations required for antagonist activity at 5-HT_3 receptors where near to full receptor occupancy would likely be required.

Since treatment with α7 nAChR agonists may benefit AD patients, who are often treated with an AChEI, we investigated the potential beneficial interaction between an AChEI and EVP-6124. The combination of previously established sub-efficacious doses of EVP-6124 (0.03 mg/kg, p.o.) and donepezil (0.1 mg/kg, p.o.) was tested and it was found that this combined treatment completely reversed the scopolamine-induced deficit in the ORT. The results of this combination study suggest that the pro-cognitive effects of AChEIs, which are dose limited because of the aforementioned adverse effects, could be enhanced if AChEIs are combined in low doses with an α7 nAChR (partial) agonist in AD patients.

We further examined the interaction of EVP-6124 and ACh at the cellular level in sustained exposure experiments that were aimed to mimick the conditions of an animal treated with EVP-6124. These data clearly illustrated that sustained exposure to a concentration of EVP-6124 below 1 nM potentiated the ACh-evoked current. Increasing the EVP-6124 concentration to 3 nM or above, caused a marked reduction of the ACh-evoked current that was attributable to receptor desensitization. Of note, a concentration lower than 3 nM of this drug alone was insufficient to activate the receptor in *in vitro* studies.

Based on the pharmacokinetic data, estimation of the maximum effective brain concentration of EVP-6124 in the ORT after a dose of 0.3 mg/kg, p.o. was less than 1 nM free drug. Therefore, the pro-cognitive effects after EVP-6124 administration appear to have occurred at brain concentrations below those that caused receptor desensitization. Furthermore, the observation that monotreatment with this drug at concentrations lower than 3 nM was insufficient to activate the receptor in *in vitro* studies, suggest an alternative mechanism of action.

Although different mechanisms can be postulated to account for the observed potentiation, the simplest and most probable model considers the co-agonistic behavior of EVP-6124 and ACh at α7 nAChRs. We were directed to a model of co-agonist activity described for tubocurarine and ACh at α3β4 nAChRs (Cachelin and Rust, 1994). In this model, a single receptor displays at least two pockets where a ligand can bind. Similarly, two molecules of ACh must bind to the α7 nAChR to activate it. Exposure to a low concentration of a high affinity ligand, such as EVP-6124, will increase the probability that a single molecule of this ligand occupies the receptor. As occupancy of the receptor by a single molecule is assumed to be insufficient to activate the receptor, exposure to such a low concentration of ligand is not expected to cause channel opening. Brief

and intermittent exposure to another ligand with lower affinity, such as ACh, will then trigger channel opening and an inward current. These steps, which correspond to the model formulated by Cachelin and Rust (1994), were summarized in Figure 7 of Chapter 5 (page 149).

Chapter 5 reported electrophysiological recordings which showed that exposure of EVP-6124 to α7 nAChRs at a sub- to low nanomolar range caused a potentiation of the ACh-evoked current. Pharmacokinetic studies indicated that these concentrations corresponded to the active doses in the behavioral tests. Therefore potentiation of ACh-evoked currents could possibly account for the pro-cognitive effects observed in animals. These data suggest that a novel mechanism of action at low partial agonist concentrations of EVP-6124 was responsible for its pro-cognitive effects.

In the ORT experiments where a natural forgetting paradigm (i.e. a 24 h delay interval) was utilized, the availability of endogenous ACh together with a low concentration of the partial α7 nAChR agonist EVP-6124 caused an improvement of cognitive performance. Following the data gathered from the electrophysiological recordings and pharmacokinetic studies, it is likely that the low concentration of EVP-6124 potentiated the α7 nAChRs for available ACh. Even more interesting, in the ORT experiments where a scopolamine-induced deficit model (and a 1h delay interval) was utilized, combined administration of sub-efficacious doses of EVP-6124 and the AChEI donepezil, completely reversed the memory deficit induced by the cholinergic antagonist scopolamine. Here, EVP-6124 was also likely to potentiate α7 nAChRs to a small extent. When subsequently combined with minimal increases of the neurotransmitter ACh, this proved to be sufficient to fully restore memory in this animal model.

The finding of a novel mechanism of action of a partial agonist acting at a concentration in the sub-nanomolar range through a co-agonism mechanism may lead to a more desirable side-effect profile than more classical approaches which dictate that the drug is dosed to full agonist concentrations. Activation of α7 nAChRs by exposure to a low agonist concentration, of a drug such as EVP-6124 utilizing this co-agonist mechanism, is expected to increase the drug safety margin, to minimize undesired interactions with other receptors, and to open new and promising therapeutic avenues in combination with classical AChEIs at lower than typically prescribed doses. Lower doses of AChEIs will most likely also result in less, or better tolerable side-effects.

Furthermore, desensitization of α7 nAChRs, and hence tolerance to these drugs, might not occur at concentrations in the sub-nanomolar range. To investigate this further a subchronic study was performed which investigated the effect of six days continuous infusion of EVP-6124 on the memory performance of rats. These studies were reported in chapter 6, and will be further discussed in the next paragraph.

EVP-6124 and desensitization

In this next study, again the cognitive enhancing effects of the partial α7 nAChR agonist EVP-6124 on natural forgetting in the ORT were assessed. The electrophysiological recordings described in chapter 5 showed that concentrations of EVP-6124 that increased the memory performance of rats in the ORT did not lead to a reduction of the ACh-evoked current after sustained exposure, and therefore did not lead to receptor desensitization. To make the translation from these *in vitro* studies to an *in vivo* model, we investigated whether the acute procognitive effect of EVP-6124 could still be observed after six days of sustained EVP-6124 exposure, at relevant concentrations, in rats. To achieve continuous exposure to the appropriate concentrations of the compound, osmotic minipumps were used.

The data from the continuous exposure experiment showed that, after six days, both plasma C_{ss} values of 0.48 ng/ml and 1.93 ng/ml significantly improved the memory performance of rats in the ORT. This suggests a lack of tolerance development to the cognition-enhancing properties of EVP-6124 for at least 6 days in these rats. The plasma concentrations required for efficacy after a single-oral dose were similar to those required under steady state conditions.

The data showed that after continuous administration of EVP-6124 in rats, no signs of behavioral desensitization (i.e. tolerance) to the cognition enhancing effects of this compound developed. This could suggest that at cognition enhancing doses, no functional desensitization of the α7 nAChRs occurred. However, this statement has to be interpreted with great caution since no long-term sustained exposure *in vitro* desensitization studies were conducted in this study. Recently it has been shown that behavioral tolerance did also not develop in schizophrenic patients after 21 days of daily treatment with EVP-6124 (Preskorn et al., 2014).

In conclusion, EVP-6124 prevented natural forgetting after an inter-trial interval of 24 h in the ORT after a single dose of 0.3 mg/kg, p.o. in male Wistar rats.

When measured plasma C_{ss} values of 0.48 and 1.93 ng/ml of EVP-6124 were maintained for six days by osmotic minipump administration, natural forgetting was also prevented. Comparable plasma concentrations were required in the single-dose and continuous infusion studies for optimal prevention of natural forgetting. Therefore, these data showed a lack of tolerance development after continuous administration of the α7 nAChR partial agonist EVP-6124 at memory enhancing doses in rats.

Recently, EVP-6124 got named encenicline, and subsequently entered phase III clinical trials for AD (ClinicalTrials.gov., NLM Identifier: NCT01969136) and schizophrenia (ClinicalTrials.gov., NLM Identifier: NCT01716975). These clinical trials are planned to be finished by the beginning of 2017.

α7 nAChR agonism

PHA 568487 and memory processes

Glutamatergic hypofunction is a well-established feature of schizophrenia and has been used extensively in modelling cognitive impairment associated with schizophrenia (CIAS) (Javitt, 2012; Meltzer et al., 2013). Acute treatment with MK-801, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, is a common way of inducing schizophrenia-like cognitive deficits in rodents (van der Staay et al., 2011). This model has been shown to possess reasonable predictive validity, with no responses to antipsychotics (e.g. risperidone, clozapine) and positive responses to putative cognitive enhancers (e.g. nicotine) mimicking responses of human patients (Brown et al., 2014). However, the AChEI donepezil consistently reversed the cognitive deficits induced by an acute dose of MK-801 (Csernansky et al., 2005; Brown et al., 2014), while it has not been successful in improving human CIAS (Keefe et al., 2008; Thakurathi et al., 2013). As schizophrenia is a chronic disorder, subchronic treatment with MK-801 might resemble CIAS better. However, less is known about the consequences of subchronic MK-801 administration in rodents.

Chapter 7 described a study in which we further explored the suitability of subchronic MK-801 administration in rodents as a preclinical model for testing possible treatments for CIAS. We assessed spatial memory in the OLT prior to, as well as at multiple time points following, subchronic MK-801 treatment in mice (0.1 mg/kg/day, i.p. for seven days). In addition, the effects of the α7 nAChR agonist PHA 568487 on memory performance were tested. Moreover, for further validation purposes, the atypical antipsychotic risperidone and the AChEI donepezil were tested in this paradigm.

Low-dose subchronic MK-801 administration induced a lasting memory deficit in mice, which persisted at least 28 days after the last drug administration. This deficit was ameliorated by the α7 nAChR agonist PHA 568487 and the AChEI donepezil. The atypical antipsychotic risperidone was not effective in improving the memory performance of the mice. Therefore, the predictive validity of the subchronic MK-801 model of CIAS is subject to the same limitation as the acute model, as revealed by the false positive response of mice to donepezil (Brown et al., 2014). Drug responses of the animals in the subchronic MK-801 model are in line with findings from acute MK-801 exposure studies, but represent an extension of the latter, since subchronic MK-801 administration resulted in enduring memory deficits, lending this model increased face validity at a functional level.

This study demonstrated that subchronic MK-801 administration can produce behaviors resembling CIAS. The ostensible absence of behaviors associated with positive symptoms in the MK-801 model (Beninger et al., 2009; Ashby et al., 2010) suggests a behavioral phenotype akin to the prodromal stages of schizophrenia, which include cognitive disturbances and early signs of negative symptomatology, but no positive symptoms (Phillips and Seidman, 2008; Kahn and Keefe, 2013).

At this stage, subchronic MK-801 exposure does not appear to be an improvement on the drug screening capacities of the acute MK-801 model of CIAS, but its enduring effects carry the added benefit of a closer resemblance to the cognitive deficits seen in human schizophrenia patients.

As a selective agonist for the α7 nAChR, 0.1 mg/kg PHA 568487 was able to improve the memory performance of the mice in the subchronic MK-801 model. This further strengthens the evidence that α7 nAChR agonism might prove beneficial in treating CIAS. Like many α7 nAChR agonists, PHA 568487 shows affinity for the 5-HT_3 receptor, although PHA 568487 shows 63 times more affinity for α7 nAChRs than for 5-HT_3 (K_i values of 44 and 2800, respectively) (Hajos and Rogers, 2010), again making the contribution of 5-HT_3 receptors to the cognition enhancing effect of the compound unlikely. Like with EVP-6124, the brain concentrations of PHA 568487 required for efficacy in the OLT (sub- to low nanomolar) are assumed to be below the concentrations required for antagonist activity at 5-HT_3 receptors where near to full receptor occupancy would likely be required.

α7 nAChR antagonism

MLA, Compound 7i and memory processes

To possibly circumvent the desensitization issue associated with sustained exposure to α7 nAChR agonists, the objective of the study described in chapter 8 was to investigate the cognition enhancing properties of low dose administration of selective α7 nAChR antagonists in rats. Interestingly, low dose monotreatment with the α7 nAChR antagonist methyllycaconitine (MLA) has sporadically been reported to improve cognition in animals (Hahn et al., 2011; Burke et al., 2014).

The α7 nAChR antagonists used for these studies were MLA and Compound 7i. Memory performance was assessed with the ORT. Furthermore, the same compounds were studied in an electrophysiological model of neuronal plasticity (LTP). In addition, microdialysis studies were performed in which hippocampal glutamate and GABA efflux were measured after administration of MLA.

It was found that both the α7 nAChR antagonists MLA and Compound 7i improved the memory performance of rats in the ORT natural forgetting paradigm at low doses (MLA: 0.003-0.1 mg/kg; Compound 7i: 0.1-1.0 mg/kg, i.p.) when administered before (but not after) the ORT learning trial. Moreover, it was found that a dose that was too high to improve memory in the natural forgetting paradigm, was also sufficient to induce a memory deficit in the 1 h retention interval ORT, an interval that normally leads to good memory performance of rats (like reported in chapter 5). Electrophysiological studies showed that low doses of MLA or Compound 7i induced a long-lasting potentiation when administered before (but not after) a weak tetanus. Microdialysis studies showed that MLA administration led to a significant increase of hippocampal glutamate efflux 30 minutes after administration that lasted up to at least 2 h after administration.

The aforementioned affinity of α7 nAChR ligands for 5-HT_3 receptors seems not to be the case with MLA. It has been shown that MLA has no affinity for 5-HT_3 receptors (Palma et al., 1996). Binding assays of Compound 7i have unfortunately not yet been published.

Chapter 8 described different hypothetical mechanisms of action that could explain the procognitive effects of low doses of α7 nAChR antagonists. In the following paragraph, these mechanisms are more extensively reviewed.

Mechanisms of action

We propose that low doses of α7 nAChR antagonists lead to alterations in the rate of desensitization or re-sensitization of the α7 nAChRs. Like explained in chapter 1, the agonist binding domain of the nAChR is at the interface between the α-subunit and the neighboring subunit (Arias, 2000), leading to as many as five functional binding sites for the homopentameric α7 nAChR. nAChRs generally require occupancy of two binding sites (by ACh or another agonist) to achieve channel opening (Cachelin and Rust, 1994), leaving in the case of α7 nAChRs three additional sites on this homopentameric receptor available for the antagonist to bind. It is plausible that from these binding sites, the desensitization of the receptor is somehow modulated.

In the allosteric model of ion channels (Figure 1), which was introduced in chapter 1, it is hypothesized that antagonists have a higher affinity for the closed conformational states (R and D) than for the open conformational state (A) (Monod et al., 1965; Karlin, 1967). The hypothesis that a low dose of an α7 nAChR antagonist could modulate the desensitized (D) conformational state of the ion channel, could mean that the desensitized (D) conformational state is modulated by low doses of antagonists to transform into the active (A) conformational state, hence enabling channel opening. Although, the exact mechanism of this transition upon antagonist binding remains speculative, this could explain our findings in the ORT, electrophysiological and microdialysis studies.

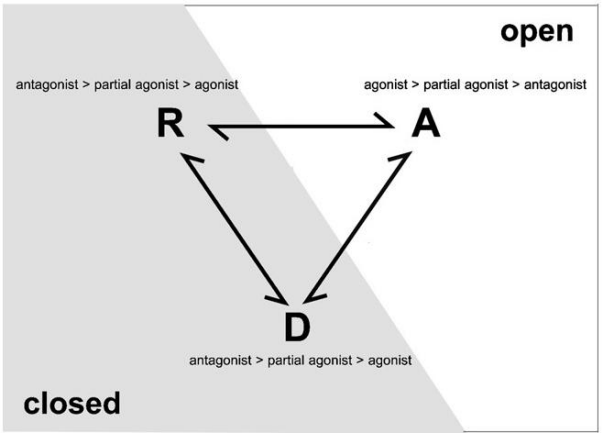


Figure 1. The minimal allosteric model for a desensitizing ligand-gated ion channel. Letters represent the active (A), resting (R) and desensitized (D) conformational states of the receptor. The receptor is closed in the R and D states. In the A state the ion channel is open and hence able to exert a biological response. The relative order of the affinities of agonists, antagonists and partial agonists of each conformational state is indicated. (Adapted from: Hogg and Betrand, 2007).

In another hypothetical mechanism of action, low doses of α7 nAChR antagonists promote α7 nAChR re-sensitization. By occupying a subset of α7 nAChRs, these receptors have an opportunity to 'recover' or re-sensitize. Subsequently, after antagonist binding, a full response to agonists (like for example ACh) can be produced after the receptors have been re-sensitized. When considering the allosteric model of ion channels again (Figure 1), this would mean that low doses of antagonists transform the conformational state of the ion channel into a state with is more susceptible to agonist binding (and hence lead to an open, active (A) state). The allosteric model predicts that antagonists have a higher affinity for the closed states, here we hypothesize that these closed conformational states are, in the presence of low concentrations of an antagonist, somehow modulated to become more susceptible to agonist binding (and therefore re-sensitize). This would mean that the low concentration of an α7 nAChR antagonist changes the relative order of the affinities agonists, partial agonists and antagonists have for each conformational state. This would give (partial) agonists an opportunity to bind more easily while the receptor is in a closed conformational state. Subsequently, upon agonist binding, the receptor can make the transition to the open (A) conformational state. Although, the exact mechanism of this modulation of a conformational state upon antagonist binding remains speculative as well, it could explain our findings in the ORT, electrophysiological and microdialysis studies.

In addition, these α7 nAChR antagonists could function as modulators, which would mean that they have different binding sites when compared to ACh or other agonists. In this explanation, the receptor recycling is somehow modulated by this mechanism, thereby preventing desensitization of the system. This would mean that the used α7 nAChR antagonists may somehow modulate desensitization from a distinct binding site. This hypothesis is rather weak, considering it has been established that both MLA and Compound 7i are competitive antagonists and, as such occupy the same binding site as ACh (Palma et al., 1996; Dani, 2001; Peng et al., 2010).

Another alternative explanation for these phenomena could be that low doses of α7 nAChR antagonists act as mild agonists, while having antagonistic properties in higher doses (mixed agonist-antagonist activity (e.g. Pick et al., 1997)). Similarly, this double role exerted by α7 nAChRs antagonists might be related to the biphasic dose-response phenomenon of hormesis, characterized by low-dose stimulation and high-dose inhibition (Calabrese, 2008). While there are, to our knowledge no published results that indicate that MLA has a mixed

agonist-antagonist activity, this effect cannot be ruled out, and remains to be investigated in future studies. Although, given the fact that the two (chemically diverse) α7 nAChR antagonists showed the same behavioral and electrophysiological profile, the chances of both compounds having such a mixed/double activity are considered small. Unless such a biological response is a hallmark response α7 nAChRs have to their antagonists. This however, also remains speculative.

Finally, in the literature, another suggested mechanism of action can be found. It has been postulated that α7 nAChR desensitization (Fujii et al., 2000; Wang and Sun, 2005; Hahn et al., 2011;) or a net decrease of receptor activity (Burke et al., 2014) has beneficial effects on cognitive performance. By administrating an α7 nAChR antagonist, the effects of α7 nAChR desensitization are mimicked and hence the observed cognitive improvement could be linked to receptor desensitization. Therefore, according to this hypothesis, the acute effects of α7 nAChR antagonists might mimic the chronic effect of α7 nAChR agonists. According to the allosteric model of ion channels, antagonist binding would keep the receptor in the closed (R and/or D) state. Therefore, a net decrease of receptor activity, following α7 nAChR antagonist administration is indeed evident. Further research is needed to investigate whether desensitization of α7 nAChRs is indeed beneficial for cognition. Since receptor desensitization would imply the build-up of tolerance to α7 nAChR ligands, we find this hypothesis unlikely, especially when considering disorders where nAChR function might be dysfunctional. Studies, in which α7 nAChR antagonists are administered in a chronic fashion (with or without agonists) would be interesting in this respect, also in conjunction with the other explanations as brought forward earlier.

Concluding remarks

Like stated in chapter 1 (and indicated in Figure 1), a partial agonist has a higher affinity for closed states (R and D) than a full agonist. This explains the antagonistic effect of a partial agonist when compared to a full agonist (Hogg and Betrand, 2007). Elaborating on this, the cognition enhancing effects of the α7 nAChR partial agonist EVP-6124 we have found in chapter 5 and 6 might be linked to the cognition enhancing effects of the α7 nAChR antagonists we have found in chapter 8. This would mean that the effects we found with EVP-6124 were related to the ability of the α7 nAChR partial agonist to actually act as an antagonist. Unfortunately, since precise measurements of the actual concentrations of the endogenous ACh in the brain at a given moment were needed in order to draw reliable conclusions about this, this remains speculative. The model of co-agonism which is summarized in Figure 7 of

Chapter 5 (page 149) still seems more plausible in this respect. Of course, both mechanisms (co-agonism and antagonism) could also have contributed to the cognition enhancing effects found for the α7 nAChR partial agonist EVP-6124.

The model of co-agonism might be worth further investigating using combinations of sub-efficacious doses of α7 nAChR antagonists with sub-efficacious doses of AChEIs or α7 nAChR partial agonists.

Like stated throughout this dissertation, the maximum response evoked by a partial agonist will depend on its efficacy. Compared to a full agonist, even when the concentration of the partial agonist is increased, the maximal response is limited. This accounts for a ceiling effect, which give partial agonists the benefit of having a larger safety margin when compared to full agonists. This makes partial agonists attractive candidates for the development of therapeutic agents (Hogg and Bertrand, 2007). In addition, Vallejo et al. (2005) reported that nicotine exposure alters the functional conformational state of the ion channel. They observed *in vitro* a slower desensitization and an enhanced sensitivity to ACh after nicotine exposure. This, in turn, leads to more easily activation upon subsequent nicotine/ACh exposure. Whether this effect also specifically applies to α7 nAChRs in response to their respective ligands remains to be investigated. In their review, Hogg and Bertrand (2007) suggest that the use of partial agonists to induce functional upregulation is likely to be associated with fewer adverse side effects when compared to full agonists.

We like to repeat the conclusion of chapter 5: Activation of α7 nAChRs by exposure to a low agonist concentration, of a drug such as EVP-6124 possibly utilizing the co-agonist mechanism, is expected to increase the drug safety margin, to minimize undesired interactions with other receptors, and to open new and promising therapeutic avenues in combination with classical AChEIs at lower than typically prescribed doses. Lower doses of AChEIs will most likely also result in less, or better tolerable side-effects.

In addition, low doses of selective antagonists for α7 nAChRs will also likely be associated with fewer adverse side effects when compared to full agonists. As such, α7 nAChR antagonists might prove very beneficial in treating cognitive dysfunction encountered in AD or schizophrenia. Indeed, while the main focus of the α7 nAChR as a target for cognition enhancement has traditionally involved agonists and positive modulators, antagonists at appropriate doses may prove to be a valuable tool for cognition enhancement in AD or schizophrenia.

References

FORUM Pharmaceuticals Inc., (October 19, 2012). Study of EVP-6124 (Alpha-7 nAChR) as an adjunctive pro-cognitive treatment in schizophrenia subjects on chronic stable atypical antipsychotic therapy. ClinicalTrials.gov. Available from: http://clinicaltrials.gov/ct2/show/NCT01716975?term=evp-6124&rank=7 (NLM Identifier: NCT01716975).

FORUM Pharmaceuticals Inc., (October 21, 2013). Study of the safety and effectiveness of two doses of investigational study drug EVP-6124 in subjects with Alzheimer's Disease. ClinicalTrials.gov. Available from: http://clinicaltrials.gov/ct2/show/NCT01969136?term=evp-6124&rank=14 (NLM Identifier: NCT01969136).

Arias, H.R. (2000). Localization of agonists and competitive antagonist binding sites on nicotinic acetylcholine receptors. *Neurochemistry International, 36*(7), 595-645.

Ashby, D. M., Habib, D., Dringenberg, H. C., Reynolds, J. N., Beninger, R. J. (2010). Subchronic MK-801 treatment and post-weaning social isolation in rats: differential effects on locomotor activity and hippocampal long-term potentiation. *Behavioural Brain Research, 212*(1), 64-70.

Beninger, R. J., Forsyth, J. K., van Adel, M., Reynolds, J. N., Boegman, R. J., Jhamandas, K. (2009). Subchronic MK-801 behavioural deficits in rats: partial reversal by the novel nitrate GT 1061. Pharmacology, *Biochemistry and Behavior, 91*(4), 495-502.

Blokland, A. (1995). Acetylcholine: a neurotransmitter for learning and memory? *Brain Research Reviews, 21*(3), 285-300.

Brown, J. W., Rueter, L. E., Zhang, M. (2014). Predictive validity of a MK-801-induced cognitive impairment model in mice: implications on the potential limitations and challenges of modeling cognitive impairment associated with schizophrenia preclinically. *Progress in Neuro-psychopharmacology and Biological Psychiatry, 49*, 53-62.

Burghaus, L., Schütz, U., Krempel, U., de Vos, R. A., Jansen Steur, E. N., Wevers, A., et al. (2000). Quantitative assessment of nicotinic acetylcholine receptor proteins in the cerebral cortex of Alzheimer's patients. *Molecular Brain Research, 76*(2), 385-388.

Burke, D. A., Heshmati, P., Kholdebarin, E., Levin, E. D. (2014). Decreasing nicotinic receptor activity and the spatial learning impairment caused by the NMDA glutamate antagonist dizocilpine in rats. *European Journal of Pharmacology, 741*, 132-139.

Cachelin, A. B., and Rust, G. (1994). Unusual pharmacology of (+)-tubocurarine with rat neuronal nicotinic acetylcholine receptors containing beta 4 subunits. *Molecular Pharmacology, 46*(6), 1168-1174.

Calabrese, E.J. (2008). Hormesis and medicine. *British Journal of Clinical Pharmacology, 66*(5), 594-617.

Carlsson, A. (1988). The current status of the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology, 1*(3), 179-186.

Counts, S. E., He, B., Che, S., Iknomovic, M. D., DeKosky, S. T., Ginsberg, S. D., et al. (2007). α7 Nicotinic receptor up-regulation in cholinergic basal forebrain neurons in Alzheimer disease. *Archives of Neurology*, *64*(12), 1771-1776.

Coyle, J. T., Price, D. L., DeLong, M. R. (1983). Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science, 219*(4589), 1184-1190.

Csernansky, J. G., Martin, M., Shah, R., Bertchume, A., Colvin, J., Dong, H. (2005). Cholinesterase inhibitors ameliorate behavioral deficits induced by MK-801 in mice. *Neuropsychopharmacology, 30*, 2135-2143.

Cummings, J. L., and Benson, D. F. (1987). The role of the nucleus basalis of Meynert and dementia: Review and reconsideration. *Alzheimer Disease and Associated Disorders*, *1*(3), 128-145.

Dani, J. A. (2001). Overview of Nicotinic Receptors and Their Roles in the Central Nervous System. *Biological Psychiatry, 49*(3), 166-174.

Dani, J. A., and Bertrand, D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews Pharmacology and Toxicology, 47*, 699-729.

Dajas-Bailador, F. A., Lima, P. A., Wonnacott, S. (2000). The α7 nicotinic acetylcholine receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampal cultures through a Ca^{2+} dependent mechanism. *Neuropharmacology, 39*(13), 2799-2807.

D'Andrea, M. R, and Nagele, R. G. (2006). Targeting the alpha 7 nicotinic acetylcholine receptor to reduce amyloid accumulation in Alzheimer's disease pyramidal neurons. *Current Pharmaceutical Design,* 12(6), 677-684.

Delacourte, A., and Defossez, A. (1986). Alzheimer's disease: tau proteins, the promoting factors of microtubule assembly are major components of paired helical filaments. *Journal of Neurological Sciences, 76*(2-3), 173-186.

D'Mello, G. D., and Steckler, T. (1996). Animal models in cognitive behavioural pharmacology: an overview. *Cognitive Brain Research, 3*(3-4), 345-352.

Forloni, G. (1996). Neurotoxicity of beta-amyloid and prion peptides. *Current Opinion in Neurology, 9*(6), 492-500.

Freedman, R., Hall, M., Adler, L. E., Leonard, S. (1995). Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. *Biological Psychiatry, 38*(1), 22-33.

Fujii, S., Ji, Z., & Sumikawa, K. (2000). Inactivation of α7 ACh receptors and activation of non-α7 ACh receptors both contribute to long term potentiation induction in the hippocampal CA1 region. *Neuroscience Letters, 286*(2), 134-138.

Galzi, J. L., Bertrand, S., Corringer, P. J., Changeux, J. P., Bertrand, D. (1996). Identification of calcium binding sites that regulate potentiation of a neuronal nicotinic acetylcholine receptor. *EMBO Journal, 15*(21), 5824-5832.

Goff, D. C., Henderson, D. C., Amico, E. (1992). Cigarette smoking in schizophrenia: relationship to psychopathology and medication side effects. *American Journal of Psychiatry, 149*(9), 1189-1194.

Gopalakrishnan, M., Molinari, E. J., Sullivan, J. P. (1997). Regulation of human α4β2 neuronal nicotinic acetylcholine receptors by cholinergic channel ligands and second messenger pathways. *Molecular Pharmacology, 52*(3), 524-534.

Hahn, B., Shoaib, M., Stolerman, I. P. (2011). Selective nicotinic receptor antagonists: effects on attention and nicotine-induced attentional enhancement. *Psychopharmacology, 217*(1), 75-82.

Hajos, M., and Rogers, B. N. (2010). Targeting α7 Nicotinic Acetylcholine Receptors in the Treatment of Schizophrenia. *Current Pharmaceutical Design, 16*(5), 538-554.

Hashimoto, K. (2009). Nausea associated with a nicotinic agonist therapy in schizophrenia. *Clinical Psychopharmacology and Neuroscience, 7*(1), 26-27.

Hogg, R. C., and Bertrand, D. (2007). Partial agonists as therapeutic agents at neuronal nicotinic acetylcholine receptors. *Biochemical Pharmacology, 73*, 459-468.

Ikonomovic, M. D., Wecker, L., Abrahamson, E. E., Wuu, J., Counts, S. E., Ginsberg, S. D., et al. (2009). Cortical α7 nicotinic acetylcholine receptor and β-amyloid levels in early Alzheimer's disease. *Archives of Neurology*, *66*(5), 646-651.

Javitt, D. C. (2012). Twenty-five years of glutamate in schizophrenia: are we there yet? *Schizophrenia Bulletin, 38*(5), 911-913.

Jonnala, R. R., and Buccafusco, J. J. (2001). Relationship between the increased cell surface α7 nicotinic receptor expression and neuroprotection induced by several nicotinic receptor agonists. *Journal of Neuroscience Research, 66*(4), 565-572.

Kahn, R. S., Keefe, R. S. E. (2013). Schizophrenia is a cognitive illness: time for a change in focus. *JAMA Psychiatry, 70*(10), 1107-1112.

Karlin, A. (1967). On the application of ''a plausible model'' of allosteric proteins to the receptor for acetylcholine. *Journal of Theoretical Biology, 16*(2), 306-320.

Keefe, R. S. E., Malhotra, A. K., Meltzer, H. Y., Kane, J. M., Buchanan, R. W., Murthy, A., et al. (2008). Efficacy and safety of donepezil in patients with schizophrenia or schizoaffective disorder: significant placebo/practice effects in a 12-week, randomized, double-blind, placebo-controlled trial. *Neuropsychopharmacology, 33*, 1217-1228.

Kem, W. R. (2000). The brain α7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). *Behavioural Brain Research, 113*(1-2), 169-181.

Lanctot, K., Rajaram, R., Herrmann, N. (2009). Review: Therapy for Alzheimer's disease: how effective are current treatments? *Therapeutic Advances in Neurological Disorders, 2*(3), 163-180.

Lee, C. Y. D., and Landreth, G. E. (2010). The role of microglia in amyloid clearance from the AD brain. *Journal of Neural Transmission, 117*(8), 949-960.

Li, Y., Papke, R. L., He, Y. J., Millard, W. J., Meyer, E. M. (1999). Characterization of the neuroprotective and toxic effects of α7 nicotinic receptor activation in PC12 cells. *Brain Research, 830*(2), 218-225.

Lilja, A. M., Porras, O., Storelli, E., Nordberg, A., Marutle, A. (2011). Functional interactions of fibrillar and oligomeric amyloid-β with alpha7 nicotinic receptors in Alzheimer's disease. *Journal of Alzheimer's Disease, 23*(2), 335-347.

Liu, Q. S., Kawai, H., Berg, D. K. (2001). β-Amyloid peptide blocks the response of α7-containing nicotinic receptors on hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(8), 4734-4739.

Lohr, J. B., Flynn, K. (1992). Smoking and schizophrenia. *Schizophrenia Research, 8*(2), 93-102.

Marin, P., Maus, M., Desagher, S., Glowinski, J., Premont, J. (1994). Nicotine protects cultured striatal neurones against N-methyl-D-aspartate receptor-mediated neurotoxicity. *Neuroreport, 5*(15), 1977-1980.

Marks, M. J., Burch, J. B., Collins, A. C. (1983). Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. *Journal of Pharmacology and Experimental Therapies, 226*(3), 817-825.

Martin-Ruiz, C. M., Court, J. A., Molnar, E., Lee, M., Gotti, C., Mamalki, A., et al. (1999). α4 but not α3 and α7 nicotinic acetylcholine receptor subunits are lost from the temporal cortex in Alzheimer's disease. *Journal of Neurochemistry*, *73*(4), 1635-1640.

Marutle, A., Gillberg, P. G., Bergfors, A., Yu, W., Ni, R., Nennesmo, I. (2013). ^{3}H-deprenyl and ^{3}H-PIB autoradiography show diferent laminar distributions of astroglia and fibrillar β-amyloid in Alzheimer brain. Journal of Neuroinflammation, 10(90), 1-15.

Meltzer, H.Y., Rajagopal, L., Huang, M., Oyamada, Y., Kwon, S., Horiguchi, M. (2013). Translating the N-methyl-D-aspartate receptor antagonist model of schizophrenia to treatments for

cognitive impairment in schizophrenia. *International Journal of Neuropsychopharmacology, 16*(10), 2181-2194.

Miyamoto, S., Miyake, N., Jarskog, L. F., Fleischhacker, W. W., Lieberman, J. A. (2012). Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Molecular psychiatry, 17*(12), 1206-1227.

Molinari, E. J., Delbono, O., Messi, M. L., Renganathan, M., Arneric, S. P., Sullivan, J. P., et al. (1998). Up-regulation of human α7 nicotinic receptors by chronic treatment with activator and antagonist ligands. *European Journal of Pharmacology, 347*(1), 131-139.

Monod, J., Wyman, J., Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. *Journal of Molecular Biology, 12*(1), 88-118.

Moon, J. H., Kim, S. Y., Lee, H. G., Kim, S. U., Lee, Y. B. (2008). Activation of nicotinic acetylcholine receptor prevents the production of reactive oxygen species in fibrillar β amyloid peptide (1-42)-stimulated microglia. *Experimental and Molecular Medicine, 40*(1), 11-18.

Nagele, R. G., D'Andrea, M. R., Anderson, W. J., Wang, H. Y. (2002). Intracellular accumulation of β-amyloid$_{1-42}$ in neurons is facilitated by the α7 nicotinic acetylcholine receptor in Alzheimer's disease. *Neuroscience, 110*(2), 199-211.

Nanri, M., Yamamoto, J., Miyake, H., Watanabe, H. (1998). Protective effect of GTS-21, a novel nicotinic receptor agonist, on delayed neuronal death induced by ischemia in gerbils. *Japanese Journal of Pharmacology, 76*(1), 23-29.

Oddo, S., and LaFerla, F. M. (2006). The role of nicotinic acetylcholine receptors in Alzheimer's disease. *Journal of Physiology-Paris, 99*(2-3), 172-179.

Palma, E., Bertrand, S., Binzoni, T., Bertrand, D. (1996). Neuronal nicotinic α7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *Journal of Physiology, 491*(1), 151-161.

Peng, X., Gerzanich, V., Anand, R., Whiting, P. J., Lindstrom, J. (1994). Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Molecular Pharmacology, 46*(3), 523-530.

Peng, Y., Zhang, Q., Snyder, G. L., Zhu, H., Yao, W., Tomesch, J., et al. (2010). Discovery of novel α7 nicotinic receptor antagonists. *Bioorganic & Medicinal Chemistry Letters, 20*(16), 4825-4830.

Phillips, L. K., Seidman, L. J. (2008). Emotion processing in persons at risk for schizophrenia. *Schizophrenia Bulletin, 34*(5), 888-903.

Picciotto, M. R., Caldarone, B. J., King, S. L., Zachariou, V. (2000). Nicotinic receptors in the brain: links between molecular biology and behavior. *Neuropsychopharmacology, 22*, 451-465.

Pick, C. G., Peter, Y., Schreiber, S., Weizman, R. (1997). Pharmacological characterization of buprenorphine, a mixed agonist–antagonist with κ3 analgesia. *Brain Research, 744*(1), 41-46.

Posadas, I., López-Hernández, B., Ceña, V. (2013). Nicotinic receptors in neurodegeneration. *Current Neuropharmacology, 11*(3), 298-314.

Prasad, C., Ikegami, H., Shimizu, I., Onaivi, E. S. (1994). Chronic nicotine intake decelerates aging of nigrostriatal dopaminergic neurons. *Life Sciences, 54*(16), 1169-1184.

Preskorn, S. H., Gawryl, M., Dgetluck, N., Palfreyman, M., Bauer, L. O., Hilt, D. C. (2014). Normalizing effects of EVP-6124, an alpha-7 nicotinic partial agonist, on event-related potentials and cognition: a proof of concept, randomized trial in patients with schizophrenia. *Journal of Psychiatric Practice, 20*(1), 12-24.

Prickaerts, J., Sik, A., van der Staay, F. J., de Vente, J., and Blokland, A. (2005). Dissociable effects of acetylcholinesterase inhibitors and phosphodiesterase type 5 inhibitors on object

recognition memory: acquisition versus consolidation. *Psychopharmacology, 177*(4), 381-390.

Rossi, A., F. Mancini, Stratta, P., Mattei, P., Gismondi, R., Pozzi F., et al. (1997). Risperidone, negative symptoms and cognitive deficit in schizophrenia: an open study. *Acta Psychiatrica Scandinavica 95*(1), 40-43.

Rowell, P. P., and Wonnacott, S. (1990). Evidence for functional activity of up-regulated nicotine binding sites in rat striatal synaptosomes. *Journal of Neurochemistry, 55*(6), 2105-2110.

Shimohama, S., Akaike, A., Kimura, J. (1996). Nicotine-induced protection against glutamate cytotoxicity. Nicotinic cholinergic receptor-mediated inhibition of nitric oxide formation. *Annals of the New York Academy of Sciences, 777*, 356-361.

Shimohama, S., Greenwald, D. L., Shafron, D. H., Akaika, A., Maeda, T., Kaneko, S., et al. (1998). Nicotinic α7 receptors protect against glutamate neurotoxicity and neuronal ischemic damage. *Brain Research, 779*(1-2), 359-363.

Sydserff, S., Sutton, E. J., Song, D., Quirk, M. C., Maciag, C., Li, C., et al. (2009). Selective α7 nicotinic receptor activation by AZD0328 enhances cortical dopamine release and improves learning and attentional processes. *Biochemical Pharmacology, 78*(7), 880-888.

Thakurathi, N., Vincenzi, B., Henderson, D. C. (2013). Assessing the prospect of donepezil in improving cognitive impairment in patients with schizophrenia. *Expert Opinion on Investigational Drugs, 22*(2), 259-265.

Toyohara, J., and Hashimoto, K. (2010). α7 nicotinic receptor agonists: potential therapeutic drugs for treatment of cognitive impairments in schizophrenia and Alzheimer's disease. *Open Medicinal Chemistry Journal, 4*, 37-56.

Vallejo, Y. F., Buisson, B., Bertrand, D., Green, W. N. (2005). Chronic nicotine exposure up-regulates nicotinic receptors by a novel mechanism. *Journal of Neuroscience, 25*(23), 5563-5572.

van der Staay, F. J., Rutten, K., Erb, C., Blokland, A. (2011). Effects of the cognition impairer MK-801 on learning and memory in mice and rats. *Behavioural Brain Research, 220*(1), 15-29.

Wallace, T. L., Callahan, P. M., Tehim, A., Bertrand, D., Tombaugh, G., Wang, S., et al. (2011). RG3487, a novel nicotinic α7 receptor partial agonist, improves cognition and sensorimotor gating in rodents. *Journal of Pharmacology and Experimental Therapeutics, 336*(1), 242-253.

Wallace, T. L., and Porter, R. H. P. (2011). Targeting the nicotinic alpha7 acetylcholine receptor to enhance cogntion in disease. *Biochemical Pharmacoly, 82*(8), 891-903.

Walstab, J., Rappold, G., Niesler, B. (2010). 5-HT_3 receptors: role in disease and target of drugs. *Pharmacology & Therapeutics, 128*(1), 146-169.

Wang, H. Y., Lee, D. H., D'Andrea, M. R., Peterson, P. A., Shank, R. P., Reitz, A. B. (2000a). β-amyloid$_{1-42}$ binds to α7 nicotinic acetylcholine receptor with high affinity: implications for Alzheimer's disease pathology. *Journal of Biological Chem*istry, *275*, 5626-5632.

Wang, H. Y., Lee, D. H., Davis, C. B., Shank, R. P. (2000b). Amyloid peptide Aβ_{1-42} binds selectively and with picomolar affinity to α7 nicotinic acetylcholine receptors. *Journal of Neurochemistry*, *75*(3), 1155-1161.

Wang, H. Y., Li, W., Benedetti, N. J., Lee, D. H. (2003). α7 Nicotinic acetylcholine receptors mediate β-amyloid peptides induced tau protein phosphorylation. *Journal of Biological Chemistry*, *278*, 31547-31553.

Wang, H., and Sun, X. (2005). Desensitized nicotinic receptors in brain. *Brain Research Reviews, 48*(3), 420-437.

Warpman, U., and Nordberg, A. (1995). Epibatidine and ABT 418 reveal selective losses of alpha4beta2 nicotinic receptors in Alzheimer brains. *Neuroreport, 6*(17), 2419-2423.

Wonnacott, S. (1990). The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends in Pharmacological Sciences, 11*(6), 216-219.

Summary

In this dissertation we studied the role of α7 nicotinic acetylcholine receptors (α7 nAChRs) in memory processes. α7 nAChR functioning was assessed on a behavioral and mechanical level in order to evaluate different possibilities to utilize this receptor subtype for treatment possibilities in different psychiatric and neurological disorders. The allosteric theory of ion channel functioning, which describes the plausible functioning of ligand-gated ion channels like α7 nAChRs, predicts that ion channel receptors can exist in different conformational states. This was described in more detail in **Chapter 1**. Furthermore, this chapter provided more background information on cognitive dysfunction, tools for preclinical research, α7 nAChRs and the effect different types of ligands can have upon ion channels.

Chapter 2 provided a comprehensive overview of the existing literature on memory functioning. Specifically, this chapter reported a brief historical overview of memory research as well as current theories and associated neuroanatomy. Human as well as animal studies were discussed. It was shown that these theories cannot fully explain the findings from empirical studies that examined the role the medial temporal lobe has in declarative memory. Therefore, in this chapter, we combined findings reported in the literature regarding theories/models of hippocampal functioning and declarative memory into a comprehensive unified framework of recognition and recall.

In **Chapter 3**, we discussed how to reliably assess different memory processes in rodent models. This is important in order to be able to effectively perform preclinical research to find memory improving drugs. This chapter focused on the memory test which was employed in several studies in this dissertation, the object recognition task (ORT). Taken together, object recognition appears to be a common ability of rodent species, but different strains have different memory capacities and sensitivities to scopolamine, individual housing leads to higher performance and performance of females is dependent on the estrous cycle phase. In addition, we discussed different pitfalls that can accompany this task.

Chapter 4 focused on how cognitive deficits as encountered in different psychiatric and neurological disorders can be modelled in rodents. Since ameliorating these dysfunctions can dramatically improve the quality of life of patients, developing treatments, or 'cognition enhancers', is a major area of interest for the pharmaceutical industry. A diverse range of animal models is being used to identify potential cognition enhancing drugs and such models can be based on pharmacological deficits, the naturally occurring aging process and/or introduction of transgenic constructs in rodents. A representative selection of rodent models of cognitive impairments in relation to common neurological and psychiatric disorders, were described in this chapter. Lastly, translation of these models to the clinic and their limitations and difficulties were described. Different validities, and to what extent these animal models satisfy these criteria, were explained as well.

The following chapters focused on cognition enhancement. The role of α7 nAChRs in memory processes and memory formation was investigated in rodents. Several studies were performed in order to further increase our understanding of the role these receptors, and their different ligands, play in enhancing memory processes.

In **Chapter 5** the first experimental study, in which a novel partial α7 nAChR agonist (EVP-6124 or encenicline) was evaluated *in vitro* and *in vivo,* was described. EVP-6124 restored memory function in scopolamine-treated rats in the ORT. Furthermore, this compound fully restored natural-, or delay-dependent forgetting in this paradigm. This improvement was blocked by the selective α7 nAChR antagonist methyllycaconitine (MLA). Furthermore, co-administration of sub-efficacious doses of the acetylcholinesterase inhibitor (AChEI) donepezil and EVP-6124, which had no effect when administered

separately, fully restored memory after a scopolamine-induced memory deficit. Accordingly, in co-application experiments of EVP-6124 with ACh, sustained exposure to EVP-6124 in functional investigations in oocytes caused desensitization at concentrations greater than 3 nM, while lower concentrations (0.3–1 nM) caused an increase of the ACh-evoked response. These actions were interpreted as representing a co-agonist activity of EVP-6124 with ACh on α7 nAChRs. The concentrations of EVP-6124 that resulted in physiological potentiation were consistent with the free drug concentrations in the brain that improved memory performance in the ORT. These data suggest that the selective partial agonist EVP-6124 improved memory performance by potentiating the ACh response of α7 nAChRs and support new therapeutic strategies for the treatment of cognitive impairment.

Chapter 6 described a study in which the (sub)chronic effects of the partial α7 nAChR agonist EVP-6124 were investigated. The objective was to assess whether tolerance to the behavioral effect, which is suggestive of receptor desensitization, for this specific drug developed. Specifically, the effect of acute and six-day continuous minipump administration of EVP-6124 on memory performance in rats was investigated. The results indicated that at EVP-6124 plasma concentrations that optimally enhanced memory in the ORT, tolerance did not develop after six days of continuous treatment. This could suggest that at cognition-enhancing doses, no functional desensitization of the α7 nAChRs occurred. However, this statement needs to be validated in future *in vitro* desensitization studies.

In **Chapter 7** an experimental preclinical pharmacological model for cognitive impairments associated with schizophrenia (CIAS) was assessed. We validated the subchronic MK-801 model. Acute N-methyl-D-aspartate receptor (NMDAR) blockade using MK-801, a non-competitive antagonist to NMDARs, is assumed to produce temporary cognitive impairments in mice similar to those seen in schizophrenic patients. Less was known, however, about the effects of subchronic MK-801 administration on cognition. The spatial memory performance of mice was assessed after subchronic treatment with MK-801. Subchronic MK-801 treatment caused lasting memory deficits, which were ameliorated by acute doses of the α7 nAChR agonist PHA 568487 and the AChEI donepezil, but were unaffected by acute administration of the atypical antipsychotic risperidone. This led to the conclusion that subchronic administration of MK-801 may lend this pharmaceutical model increased face

validity, while its resemblance to prodromal schizophrenia makes it suitable for screening new CIAS treatments.

Chapter 8 described mechanistic and behavioral studies which investigated an alternative strategy to enhance memory performance by utilizing α7 nAChRs. The objective was to investigate the cognition enhancing properties of low dose administration of selective α7 nAChR antagonists in rats. The compounds used for these studies were MLA and Compound 7i. Interestingly, we found that low doses of MLA and Compound 7i improved object recognition memory at a 24 h retention interval. Conversely, higher doses impaired the memory performance at a shorter 1 h retention interval. In addition, the same compounds were studied in a model of neuronal plasticity, long-term potentiation (LTP). We demonstrated that pre-tetanus low-dose administration of MLA or Compound 7i produced a longer lasting potentiation. Moreover, microdialysis studies showed that low dose MLA administration substantially increased hippocampal glutamate efflux which has been found to be related to object memory processes. In summary, while the main focus of the α7 nAChR as a target for cognition enhancement lies on agonists and positive modulators, antagonism of these receptors at low doses might also prove to be a valuable tool for cognition enhancement in Alzheimer's disease or schizophrenia.

To summarize, in this dissertation the role of α7 nAChRs in memory processes was studied on a behavioral and mechanical level. We specifically emphasized on the role of α7 nAChRs in Alzheimer's disease and schizophrenia. α7 nAChR ligands might prove to be very beneficial to ameliorate cognitive dysfunction as encountered in both Alzheimer's disease and schizophrenia. Based on our findings we conclude that the most promising α7 nAChR ligands for cognition enhancement are selective partial agonists and antagonists at very low concentrations. We have put the idea forward that both ligand types might actually work via the same mechanism, i.e. modulation of the conformational states of the ion channel. This could lead to modulation of the desensitized state, i.e. less desensitization of the receptor or easier transformation to an active state. Alternatively, antagonism might also transform the conformational closed states of the ion channel into a state with is more susceptible to agonist binding, given that the antagonist concentration is low enough. This however, remains speculative. In conclusion, the findings of α7 nAChR partial agonists and antagonists to enhance α7 nAChR functioning might shed a new light upon the allosteric theory of ion channel functioning.

Samenvatting

In dit proefschrift hebben we de rol van α7 nicotine acetylcholine receptoren (α7 nAChRs) in geheugenprocessen onderzocht. We hebben het functioneren van de α7 nAChRs bestudeerd op een gedragsmatig en mechanistisch niveau. Het doel hiervan was te evalueren welke mogelijkheden er zijn om dit receptor subtype als farmacologische behandeling voor verschillende psychiatrische en neurologische aandoeningen te gebruiken. Om het mogelijke mechanisme achter ion kanalen (zoals de α7 nAChRs) te beschrijven wordt de allosterische theorie van ion kanalen gebruikt. Deze theorie voorspelt dat ion kanaal receptoren zich in verschillende conformationele toestanden kunnen bevinden.
Een gedetailleerde beschrijving van dit model werd gegeven in **Hoofdstuk 1**. Verder gaf dit hoofdstuk meer achtergrond informatie omtrent cognitieve stoornissen, methodieken van preklinisch onderzoek, α7 nAChRs en het effect dat verschillende typen liganden op deze ion kanalen kunnen uitvoeren.

Hoofdstuk 2 gaf een uitgebreid overzicht van de bestaande literatuur omtrent het functioneren van ons geheugen. In dit hoofdstuk werd een kort historisch overzicht van geheugenonderzoek gegeven. Verder werden de huidige theorieën en bijbehorende neuroanatomie van geheugenprocessen uitvoerig beschreven. Er werd ingegaan op zowel humane- als dier-experimentele studies. Er werd aangetoond dat de huidige theorieën de bevindingen uit empirische studies naar de rol van de mediale temporale kwab in geheugenprocessen, niet volledig kunnen verklaren. Om deze reden hebben we in dit hoofdstuk bevindingen en modellen uit de literatuur omtrent hippocampaal functioneren

en geheugenprocessen gecombineerd in een uitgebreid model. Dit model beschrijft gedetailleerd hoe de processen van herinneren en herkennen met elkaar verbonden zijn.

Hoofdstuk 3 beschreef hoe verschillende geheugenprocessen in knaagdiermodellen op een betrouwbare manier kunnen worden bestudeerd. Dit is van groot belang in preklinisch onderzoek waar gezocht wordt naar nieuwe medicijnen ten behoeve van het verbeteren van geheugenprocessen in bijvoorbeeld patiënten met de ziekte van Alzheimer of schizofrenie. Dit hoofdstuk richtte zich voornamelijk op de object herkenningstaak (ORT). De ORT is een knaagdiertest om geheugenprocessen te meten. Deze test werd gebruikt in verschillende studies die in dit proefschrift staan beschreven. Samengevat kunnen we concluderen dat object herkenning een gemeenschappelijk vermogen van verschillende knaagdiersoorten is, dat er verschil is in geheugencapaciteiten en reacties op scopolamine tussen knaagdierstammen, dat het individueel huisvesten leidt tot betere geheugenprestaties in deze taak en dat de prestatie van vrouwelijke knaagdieren afhankelijk is van de oestrus cyclus. Verder beschreef dit hoofdstuk verschillende aandachtspunten welke van belang zijn wanneer gebruik wordt gemaakt van deze test.

In **Hoofdstuk 4** werd ingegaan op de vraag hoe cognitieve stoornissen, welke ondervonden worden in verschillende psychiatrische en neurologische aandoeningen, vertaald kunnen worden in een knaagdiermodel. Het verbeteren van deze cognitieve stoornissen kan de kwaliteit van leven van patiënten drastisch verhogen. Het ontwikkelen van farmacologische stoffen om cognitieve stoornissen te verbeteren is daarom ook één van de interessegebieden van de farmaceutische industrie. Om dergelijke stoffen te ontwikkelen en te identificeren worden verschillende knaagdiermodellen gebruikt. Dergelijke modellen kunnen gebaseerd zijn op farmacologisch-geïnduceerde cognitieve verslechtering, het natuurlijke verouderingsproces en/of het creëren van transgene knaagdieren. Dit hoofdstuk geeft een overzicht van verschillende knaagdiermodellen voor cognitieve stoornissen. Verder werd de relatie van ieder knaagdiermodel tot het specifieke ziektebeeld waar ze voor ontworpen zijn beschreven. Aan het eind van dit hoofdstuk werd de vertaalslag van deze modellen naar de kliniek behandeld. Daarnaast werden de beperkingen en moeilijkheden van knaagdiermodellen voor humaan onderzoek beschreven. Verschillende vormen van validiteit en in hoeverre deze knaagdiermodellen hieraan voldoen, werden tevens toegelicht in dit hoofdstuk.

De volgende hoofdstukken richtte zich op farmacologische stoffen om cognitieve stoornissen te verbeteren. De rol van α7 nAChRs in geheugenprocessen is onderzocht in knaagdieren. Verschillende studies zijn uitgevoerd met als doel beter in kaart te brengen welke rol deze receptoren, en stoffen welke hierop inwerken, spelen bij het verbeteren van geheugenproccessen.

In **Hoofdstuk 5** werd de eerste experimentele studie met een nieuwe partiële α7 nAChR agonist (EVP-6124 or encenicline) beschreven. Deze stof is zowel *in vitro* als *in vivo* onderzocht. We lieten zien dat EVP-6124 het geheugen van ratten volledig kon herstellen in een farmacologisch model waar een geheugenverslechtering geïnduceerd werd met scopolamine in de ORT. Verder vertoonde de stof ook een geheugenverbeterend effect in het natuurlijke-, of tijdsinterval-afhankelijke vergeetmodel in deze taak. Geheugenverbetering kon tegengegaan worden door tevens de selectieve α7 nAChR antagonist methyllycaconitine (MLA) toe te dienen aan de ratten. Daarnaast vonden we dat het gelijktijdig toedienen van sub-effectieve doseringen van de acetylcholinesterase remmer (AChEI) donepezil en EVP-6124, welke geen effect hadden wanneer deze apart werden toegediend, het geheugen van ratten volldig kon herstellen na een scopolamine-geïnduceerde verslechtering. Overeenkomstig lieten we in *in vitro* studies zien dat een langdurige blootstelling aan EVP-6124, receptor desensitisatie induceerde bij concentraties boven de 3nM. Dit werd aangetoond middels de reactie die acetylcholine (ACh), na EVP-6124 blootstelling, opwekte bij de receptoren. Lagere concentraties van EVP-6124 (0,3-1 nM) leidde juist tot een verhoging van de reactie welke door ACh werd opgewekt, en daarmee dus tot receptor potentiëring. Deze bevindingen werden geïnterpreteerd als zogenaamde co-agonist activiteit van ACh en EVP-6124 op α7 nAChRs. De *in vitro* EVP-6124 concentraties welke in een receptor potentiëring resulteerden waren overeenkomstig met de vrije stof concentraties in het brein welke het geheugen verbeterden in de ORT. Deze bevindingen suggereren dat de selectieve partiële α7 nAChR agonist EVP-6124, het geheugen verbeterde door middel van het potentiëren van de actie van ACh op de α7 nAChRs. Deze bevindingen ondersteunen nieuwe farmaceutische strategieën om cognitieve verslechtering te behandelen.

Hoofdstuk 6 beschreef een experiment waarin de (sub)chronische effecten van de selectieve partiële α7 nAChR agonist EVP-6124 werden onderzocht. Het doel was te onderzoeken of tolerantie voor het geheugenverbeterende effect van EVP-6124 optrad. Het ontstaan van tolerantie zou kunnen betekenen dat

receptor desensitisatie heeft plaatsgevonden. Het effect van continue toediening van EVP-6124 door middel van osmotische minipompjes, op de geheugenprestaties van ratten werd onderzocht. De resultaten lieten zien dat bij plasma concentraties welke het geheugen van de ratten in de ORT optimaal verbeterden, geen tolerantie optrad na zes dagen continue blootstelling aan EVP-6124. Deze bevindingen suggeren dus dat bij cognitie-verbeterende doseringen van EVP-6124, een functionele desensitisatie van de α7 nAChRs uitblijft. Echter, dergelijke conclusies dienen ondersteund te worden met toekomstige *in vitro* desensitisatie studies.

In **Hoofdstuk 7** werd een experimenteel preklinisch farmacologisch model gevalideerd voor de cognitieve verslechtering welke geassocieerd wordt met schizofrenie. Acute N-methyl-D-aspartate receptor (NMDAR) blokkering wordt verondersteld tijdelijke cognitieve verslechtering te induceren in muizen welke vergelijkbaar is met die in schizofrenie patiënten. Acute NMDAR blokkering kan worden gerealiseerd door toediening van MK-801, een non-competatieve NMDAR antagonist. Echter minder was bekend omtrent de effecten van subchronisch blootstelling aan MK-801 op cognitieve vaardigheden. De spatiële geheugenprestatie van muizen werd bestudeerd na een subchronische behandeling met MK-801. Subchronische toedieningen van MK-801 leidde tot langdurige geheugenverslechtering. Deze verslechtering kon volledig worden hersteld door de acute toediening van de α7 nAChR agonist PHA 568487 en de AChEI donepezil. De geheugenverslechtering kon echter niet worden verbeterd door de acute toediening van het atypische antipsychoticum risperidone. Deze bevindingen leiden tot de conclusie dat het subchronische MK-801 model een betere *face validity* bezit dan het acute MK-801 model doordat de geheugenverslechtering langduriger van aard is. Dit model vertoont gelijkenis met de prodromale fase van schizofrenie waarin voornamelijk cognitieve en negatieve symptomen zich voordoen. Derhalve kan het subchronische MK-801 model een uitkomst bieden bij het onderzoek naar nieuwe behandelingen voor cognitieve verslechtering welke wordt geassocieerd met schizofrenie.

Hoofdstuk 8 beschreef mechanistische- en gedragsstudies welke een alternatieve strategie voor cognitieve verbetering, door gebruik te maken van α7 nAChRs, onderzochten. Het doel was te onderzoeken of lage doseringen van selectieve α7 nAChR antagonisten konden leiden tot geheugenverbetering in ratten. De selectieve α7 nAChR antagonisten die gebruikt werden in deze studies waren MLA en Compound 7i. Er werd ontdekt dat erg lage doseringen

van MLA en Compound 7i de geheugenprestatie van ratten in de ORT konden verbeteren in het natuurlijke-, of tijdsinterval-afhankelijke vergeetmodel (24 uur interval). Tevens werd gevonden dat hogere doseringen van deze stoffen het geheugen van ratten verslechterden in een 1 uur interval in deze taak. Dit interval leidt normaliter tot een goede geheugenprestatie. Daarnaast hebben we dezelfde stoffen getest in een model voor neuronale plasticiteit, zogenaamde lange-termijn potentiëring (LTP). We hebben laten zien dat een toediening van een lage dosering MLA of Compound 7i leidde tot een langdurigere potentiëring. Bovendien bleek uit bevindingen van microdialyse studies, dat het toedienen van lage doseringen MLA een substantiële verhoging van de neurotransmitter glutamaat in de hippocampus tot gevolg had. Dit proces wordt in de literatuur geassocieerd met object geheugenprocessen. Terwijl de voornaamste aandacht voor de α7 nAChR om cognitie te verbeteren uitgaat naar agonisten en positieve modulatoren voor deze receptor, kunnen de antagonisten voor deze receptoren in erg lage doseringen weleens waardevol blijken voor het verbeteren van cognitieve processen in de ziekte van Alzheimer en schizofrenie.

In dit proefschrift werd de rol die α7 nAChRs in geheugenprocessen spelen onderzocht op een gedragsmatig en mechanistisch niveau. Hierbij werd voornamelijk de nadruk gelegd op de rol van deze receptoren in de ziekte van Alzheimer en schizofrenie. α7 nAChR liganden kunnen zeer waardevol blijken voor het verbeteren van cognitieve verslechtering welke aangetroffen wordt bij zowel de ziekte van Alzheimer als schizofrenie. Op basis van onze bevindingen concluderen we dat de meest veelbelovende α7 nAChR liganden voor cognitieve verbetering, selectieve partiële α7 nAChR agonisten en antagonisten in erg lage concentraties zijn. We hebben het idee naar voren gebracht dat beide typen liganden weleens via hetzelfde mechanisme zouden kunnen werken, namelijk via modulatie van de conformationele toestanden van het ion kanaal. Dit zou kunnen leiden tot een modulatie van de gedesensitiseerde conformationele toestand, dat wil zeggen dat er minder receptor desensitisatie optreedt of dat de receptor gemakkelijker naar een actieve conformationele toestand transformeert. Anderzijds zou antagonisme de conformationele gesloten toestand van het ion kanaal kunnen transformeren naar een staat waarin het ion kanaal meer vatbaar is voor binding van een agonist. Dit geschiedt alleen indien de concentratie van de antagonist laag genoeg is. Dit blijft echter speculatief. Ten slotte, de bevindingen dat zowel α7 nAChR partiële agonisten en antagonisten het functioneren van de α7 nAChRs kunnen verbeteren, kan

een nieuw licht werpen op de allosterische theorie omtrent het functioneren van ion kanalen.

Valorisation Addendum

Relevance & Audiences

Cognitive dysfunction is a feature often encountered in a broad spectrum of neurological and psychiatric conditions. These cognitive deficits are most commonly found in patients suffering from Alzheimer's disease (AD), dementia, schizophrenia, stroke, attention deficit hyperactivity disorder (ADHD) and aging. Ameliorating these dysfunctions can dramatically improve the quality of life of patients. Hence, developing treatments, or 'cognition enhancers' (nootropics), is imperative in this respect. The studies described in this dissertation have mainly focused on cognitive impairments as encountered in AD and schizophrenia. The best known example is probably AD, in which memory problems are the major hallmark which place a tremendous burden on the quality of life of patients.

AD is the most common form of elderly dementia. In 2010, the World Health Organization estimated that 35.6 million people worldwide suffered from dementia. Mainly due to the increasing aging of the world population, over 115 million people will be suffering from this disease by the year 2050. A substantial majority of these cases will be due to Alzheimer's pathology. 40% of AD patients will require intensive nursing home care, which will lead to a massive economic burden on social health care resources. There are five approved cognition enhancers for AD on the market which either modulate the cholinergic system by inhibition of acetylcholinesterase (AChE) or presumably reduce glutamate

neurotoxicity via NMDA antagonism. However, all have limited efficacy and severe side-effects. The etiology of AD is not well understood and current treatment for AD is ineffective both in combating cognitive deficiencies as well as in stopping disease progression. Thus, AD represents one of the major unmet medical needs for our lifetime.

The economic burden of AD patient care is enormous. In 2014, the direct costs to the American society of caring for those with AD will total an estimated $214 billion, including $150 billion in costs of medical care. This is expected to increase to over $1 trillion by 2050 in the US alone. Extension of institutionalization of only one year will already greatly reduce these future costs. Cognition enhancers can contribute to this extension of institutionalization by restoring cognitive functioning and hence independent living.

Schizophrenia is a disabling psychiatric disorder that affects about 1% of the population worldwide. It has a major negative influence on the quality of life of patients. The disease is associated with a significant and long-lasting health, social and financial burden, not only for the patients (60% is unemployed), but also for families, other caregivers and the wider society. The extent of cognitive deficiencies is the best predictor of functional outcome when compared to the positive and negative symptomatology. At the same time, the cognitive demands of today's society increase due to the increased flow of information via internet, television and cell phones in daily life as well as at work. However, the current available antipsychotic drugs are not effective in treating cognitive symptoms. Development of treatment for cognitive impairments will have a substantially beneficial effect on the functional outcome of patients, making it possible again to read a book or have a (telephone) conversation. Such a treatment will considerably improve the quality of life of the patient and will additionally have a significant social and economic impact, if the patients can return to work. In addition to the social and economic benefits, the effect of this return to independence would considerable improve the patient's self-esteem. If such a cognition enhancer is available, it could be a major breakthrough.

In addition, most neurodegenerative and psychiatric disorders have cognitive impairments for which no treatment exists. This explains the need and still ongoing search for better cognition enhancers. α7 nicotinic acetylcholine receptor (α7 nAChRs) ligands are relatively new drugs in this respect.

In this dissertation I showed that the cognitive performance of rodents can be improved by utilizing the α7 nAChR system.

In summary, I showed:

1. that α7 nAChR partial and full agonists have an acute positive effect on learning and memory;
2. that continuous exposure to an α7 nAChR partial agonist did not lead to behavioral tolerance for at least up to 6 days of treatment;
3. that combined treatment with suboptimal doses of an α7 nAChR partial agonist and an AChE inhibitor, which showed no effect when administered separately, led to improved cognitive performance;
4. that low dose administration of α7 nAChR antagonists led to improved memory formation, hippocampal glutamate efflux and cognitive performance.

It has been shown that α7 nAChRs are largely unaffected in the AD brain. Hence, different α7 nAChR agonists have been investigated for their potential to improve memory and attention disorders encountered in AD. A major drawback of α7 nAChRs is that they show rapid desensitization following repeated exposure to full agonists, thereby quickly inducing tolerance in patients taking medicines that agonistically target the α7 nAChRs. The studies in this dissertation indicated that using partial agonists, combinations of suboptimal doses of partial agonists and AChE inhibitors, and even antagonists might prove to be beneficial in AD. It is unlikely that these strategies will lead to the development of tolerance to the drugs.

It has been hypothesized that amyloid plaques in AD brains are actually the lysis remnants of degenerated, beta amyloid peptide 42 (Aβ42)-overburdened neurons. The most vulnerable neurons for Alzheimer pathology, in particular the toxic plaque formation, appear to be those that abundantly express the α7 nAChR, and internalization of Aβ42 appears to be facilitated by the high-affinity binding of Aβ42 to the α7 nAChRs on neuronal cell surfaces. This is followed by endocytosis of the resulting complex and its accumulation within the lysosomal compartment thus results in amyloid plaque formation eventually. Elaborating on this, treatment with α7 nAChR ligands might reduce the disease progression via occupying these receptors and hence prevent Aβ42 from binding to the receptor. Therefore, these drugs may make the brain more resistant against the destructive effects of Aβ depositions. To summarize, α7 nAChR ligands might both directly improve learning and memory, while they also slow down actual disease progression.

Unravelling the working mechanisms of the α7 nAChRs will not only benefit AD. α7 nAChRs have been shown to be involved in other psychiatric conditions like schizophrenia and ADHD. For example in schizophrenia, preclinical and clinical studies have shown that α7 nAChR ligands were able to normalize the auditory gating deficit in both rodent models and in schizophrenic patients. In addition, when considering the cognitive symptoms of schizophrenia and the well-established pro-cognitive effect α7 nAChR ligands have on cognition, it is likely that α7 nAChR ligands are beneficial as an add-on therapy in the treatment of schizophrenic patients. Also in ADHD, it is assumed α7 nAChR ligands can improve the symptoms of this disorder via stimulating working memory and attention processes. Therefore, α7 nAChR ligands can be expected to also be beneficial in these disorders. Until an actual cure is found for these disorders, symptomatic treatment will stay the most desirable solution for these patients. Relieving the symptoms of these disorders will already greatly improve the quality of life of patients.

Furthermore, similar ion channels like $GABA_A$, glycine and $5\text{-}HT_3$ would most likely function via a similar mechanism. This opens up a brand new view on these receptor subtypes to develop better treatments for a plethora of different neurological and psychiatric conditions.

Clearly, the results from these studies are of interest for the pharmaceutical industry. Developing new (symptomatic) treatments for AD, schizophrenia or other neurological or psychiatric disorders in which cognition is impaired, is commercially interesting for industry. Actually industry is the stakeholder having the financial resources and infrastructure organization to bring a new drug to the market. Of note, part of research related to the drug EVP-6124 as described in this dissertation is a collaboration with its owner FORUM Pharmaceuticals (Boston, USA). In addition, research on the α7 nAChR antagonist Compound 7i is a collaboration with Intra-Cellular Therapies, Inc. (New York, USA). EVP-6214 (or: Encenicline) is currently in clinical phase III trials for AD and schizophrenia.

In summary, the results of the studies described in this dissertation are of importance to patients, their family and caretakers, governments (reduction in medical costs) and pharmaceutical industries.

Activities/Products & Innovation

Actual products which can be derived from the studies in this dissertation are improved pharmacological treatments for AD and schizophrenia patients. A big advantage of treatment with α7 nAChR partial agonists and antagonists is that desensitization of the receptors, and therefore tolerance to the drugs, does not develop. The development of tolerance is a known phenomenon for selective full agonists, and this resulted in the termination of most α7 nAChR programs in different pharmaceutical companies. Since α7 nAChRs are still abundantly present in the brains of AD patients, the target still seems promising. Partial agonists and antagonists of the α7 nAChR could therefore prove to be very beneficial in the functional outcome of AD patients.

Furthermore, parallel studies with 'follow-up' compounds could be patented for new indications, like for example schizophrenia and ADHD. This opens up new possibilities for specific blocking/mild activation of α7 nAChRs.

In conclusion, the findings of α7 nAChR partial agonists and antagonists to enhance α7 nAChR functioning and therefore learning and memory shed a new light on the α7 nAChR as a target for cognition enhancement and even neurodegenerative pathology reduction.

Dankwoord/Acknowledgements

Het is zover, hetgeen waar ik toch wel erg naar uit gekeken heb, het schrijven van mijn dankwoord! Een bezigheid die van buitenaf als erg luchtig wordt beschouwd maar in werkelijkheid toch wel de nodige aarzelingen bij mij oproept. Dit komt met name omdat dit het hoofdstuk is wat eigenlijk iedereen als eerste leest. Ik hoop dan ook van harte dat ik hier niemand vergeet. Zoals Stelling 8 bij dit proefschrift al aangaf: “Working together is succes”. Een proefschrift komt tot stand door samenwerkingen. Er zijn een aantal personen in het bijzonder die een belangrijke bijdrage hebben geleverd, en tot deze personen wil ik me aan het einde van dit proefschrift dan ook graag nog even richten.

Ten eerste natuurlijk Dr. J. Prickaerts, mijn copromotor, welke mij een kans gaf, in mij geloofde en me altijd wist te motiveren net een stukje verder te gaan.
Beste Jos, (Dr. B.M. Prickaerts), het lijkt zo kort geleden dat ik je kantoor binnen stapte voor een stage en eigenlijk geen idee had wat ik wilde doen, enkel wat ik leuk en interessant vond. Jij hebt me het licht laten zien en de kennis die je aan mij hebt overgedragen is voor mij van onschatbare waarde. Ik ben je daarvoor dan ook ontzettend dankbaar. Je hebt me altijd kunnen inspireren en motiveren. Ik kan je niet genoeg bedanken voor het geloof dat je in me had en de vrijheid die je me hebt gegeven om dit mooie proefschrift te kunnen maken. *“See the deed, get it done”* een uitstekend advies wat ik nooit zal vergeten. Tevens wil ik je nog bedanken voor alle gelegde contacten en plezier wat we hebben gehad op de vele reisjes die ik in de afgelopen 4 jaar met jou heb mogen maken. Jos, ontzettend bedankt!!!

Prof. Dr. H.W.M. Steinbusch, mijn promotor. Beste Harry, graag wil ik u bedanken voor de mogelijkheid om binnen de School for Mental Health and Neuroscience te mogen promoveren en het nauwgezet in de gaten houden van mijn voortgang.

Tevens wil ik graag de leden van de beoordelingscommissie bedanken voor de vakkundige en positieve beoordeling van mijn proefschrift. Prof. Dr. Therese van Amelsfoort, Prof. Dr. Marc de Baets, Dr. Arjan Blokland, Dr. Ernesto Fedele, en Prof. Dr. Huib Mansvelder: bedankt!/grazie!

Mijn paranimfen: Britt en Pim, jullie komen hierna uiteraard nog aan bod. Maar bij deze wil ik jullie alvast hartelijk danken om op het moment supreme aan mijn zijde te staan. Ik kijk erg uit naar jullie proefschriften, en hoop dat we nog een tijdje samen mogen werken.

Dr. Blokland, beste Arjan, graag wil ik jou nog apart bedanken voor alles wat je voor me hebt gedaan de afgelopen jaren. Ook de vele malen dat we elkaar in de trein naar Beek troffen en samen naar huis gefietst zijn vond ik altijd prettig en verhelderend. Vooral het tripje naar het mooie La Rochelle zal ik nooit vergeten!

I would also like to thank Dr. Lawrence Wennogle and the other nice people over at Intra-Cellular Therapies, Inc. in New York.
Dear Larry, thanks a lot for giving me the opportunity to work at ITI and for taking such good care of me. I am very grateful for the great atmosphere and high scientific standards I was able to experience over at ITI. Also, thanks a lot for the great canoe trip and Thanksgiving dinner me and Britt were invited to. And of course, thank you Robin for your hospitality and kindness!
In addition, a special thanks to the other nice people and great scientists over at ITI: Joseph, Gretchen, Allen, Stephanie, Youyi, Peng, Jennifer, Pavel and all the others. Thanks a lot!!

Furthermore, I would like to thank Dr. Holly Moore and Benjamin Inbar of the New York State Psychiatric Institute. Dear Holly and Benjamin, thanks a lot for making it possible for me to perform the behavioral experiments in your labs. I hope our paths will cross again, maybe even in the Netherlands!

A special thanks to Dr. Robert E. Clark of the University of California, School of Medicine in San Diego, for giving me permission to use his great graphic design

'Hippocampal Neurons' for the cover of this dissertation. I really appreciate your kindness and permission.

Dat brengt mij bij MHeNS en mijn directe collega's van de afgelopen jaren. Ten eerste natuurlijk de In-Vivo Kern, de Mood & Cognition Group. We love drugs! Wat hebben we een mooie tijd gehad. Work hard, party hard! Mede dankzij jullie hulp en steun is dit proefschrift tot stand gekomen. Bedankt Annerieke, Caroline, Jerome, Kris, Marlies, Olga, Sven en Tim!

Eva, bedankt voor alles gedurende de periode dat we samen werkten, van goede gesprekken tot bizarre gehaktdagen. We wisten elkaar altijd weer te motiveren. We zullen elkaar snel wel weer ergens treffen, of het nu in Parijs, Krakau of Manhattan is. O ja, Eva's oogBollen-test wordt hier nog altijd toegepast... Bedankt Eva!

Jochen en Pim (& Ricky Martin!), jullie zijn fijne collega's! Bedankt voor alle hulp, brainstorm-sessies en do-mi-bo's! Met jullie door Brazilië reizen was een geweldig avontuur. The Pantanal experience, zwemmen met piranha's en de piranha bloed/bier-test zijn niet te overtreffen. Mannen bedankt, op naar de VS!

Dagmar (Elektrotechniek!), bij deze dan mijn boekje. Ook ik vond het erg prettig om experimenten met jou uit te mogen voeren. Ik ben erg blij met de publicatie die eruit voortgekomen is en wacht met spanning op de tweede. Succes met je verdere carrière! Maar jou kennende, komt dat dik in orde.

Wiel, het beste paard van stal. Ik ben blij dat ik in het begin van mijn werkzaamheden binnen MHeNS bij jou op kantoor heb mogen zitten. Je was altijd daar voor tips & tricks, vergeten medicatie of een goed gesprek met een kop koffie. Wiel bedankt dat je me af en toe met de neus weer de juiste kant op hebt gezet!

Ook de studenten die mij gedurende mijn promotie onderzoek hebben geholpen wil ik van harte bedanken. Bedankt/Thanks: Dila, Stoyo, Miro, Anna en Thecla!

Dan de overige MHeNS'en die tijdens mijn promotietijd op wat voor manier dan ook hebben bijgedragen aan een succesvolle afronding, van interessante gesprekken tot praktisch hulp, in alfabetische volgorde: Alejandro, Ali, Artemis, Bart, Caroline, Celine, Daniel, Ehsan, Eva van Donkelaar, Evelien, Evi (Groetjes

terug!), Fabien, Gerard, Gunther, Jo, João, Jody, Julie, Katleen, Kimberly, Koen, Leonidas, Lisa, Maarten, Mark, Nicole, Pablo, Ramona, Remco, Rianne, Roy, Sandra, Sarah, Simone, Youssef, en alle anderen. It was great working together, thanks a lot!

And then we have the terrific bunch of people I keep running into on conferences and have had a lot of fun with: Kris, Lars and Fede! Thanks a lot for all the good times in San Diego, Washington, La Rochelle and where not. I'm sure we'll meet again in Chicago!

My good friends from Brazil, thank you all! We had a great time in Ribeirão Preto and in Maastricht! I hope to visit you all again soon to spend some more time in the Brazilian sun! Special thanks to Davi, Leandro, Vivian, Ligia and Melina!

Dan zijn er natuurlijk onze analisten: Hellen, Denise en Marjan, welke ik hartelijk wil bedanken voor alle technische ondersteuning en hulp bij experimenten.
Tevens gaat mijn dank uit naar de medewerkers van het CPV, welke de dieren die ik gebruikt heb in deze studies hebben verzorgd en ervoor zorgden dat alles in de dierverblijven in orde was. Verder gaat mijn dank natuurlijk uit naar alle ratten en muizen zelf, zonder hen geen nieuwe medicijnen!

I would also like to thank Gerhard and Dorothy from FORUM Pharmaceuticals (former: EnVivo Pharmaceuticals). Thanks a lot for the great collaboration over the years. I'm really happy that it resulted in two nice publications which formed an important foundation for this dissertation. Apart from that, the studies I did for you triggered my interest in α7 nAChRs and ion channels in general. Gerhard and Dorothy, thanks a lot! Fingers crossed for EVP-6124!

From the US back to Europe. I would like to thank Daniela Puzzo for performing the electrophysiological (LTP) studies, Ernesto Fedele for the microdialysis studies and Daniel Bertrand for all the *in vitro* work on EVP-6124. These collaborations have really improved the publications and hence also my dissertation. I'm sure we will collaborate in the future again!

Dat was denk ik zo ongeveer iedereen die op (more or less) professioneel vlak een belangrijke bijdrage heeft geleverd aan dit proefschrift. Maar de werkomgeving is zeker niet alles. Een sterke sociale achterban is minstens zo belangrijk...

Daarom wil ik allereerst dan ook mijn geweldige en gestoorde vriendengroep bedanken, de ‘geen-januari crew’. In alfabetische volgorde zijn dat natuurlijk: Cyril, Harald, Jorick, Koen, Lynn, Roy, Stef, Sven, Tom, Walter en aanhang. Jullie hebben allemaal (wellicht zelfs zonder het te weten) op jullie eigen manier een bijdrage geleverd aan dit proefschrift. Als klankbord, praktijk voorbeeld en uitlaatklep hebben jullie me stuk voor stuk door de afgelopen 4 jaar heen gesleept. Monsters, bedankt! Ik verheug me op de volgende bijeenkomst!

Jorick, tot jou wil ik me graag nog even apart richten. Altijd daar voor een goed gesprek, in super tijden en in diep ellendige periodes. Altijd fijn om ‘even’ een uur te bellen. Jij hebt me ondertussen al zo vaak geholpen, dat een hele pagina in dit boekje het niet zou kunnen dekken. Ik ben ontzettend blij met onze vriendschap. Marieke ook bedankt voor de gezelligheid en gastvrijheid (dr gaat ook niks in zo’n fles hè ;). Een nu zelfs een nieuwe aanwinst in de family! Siem, ik ben zeer blij dat ik je mag gaan zien opgroeien. Zorg goed voor die oudjes!

Dan zijn daar oud-studiegenoten die gelukkig zijn blijven plakken: Kenny, Pim en Rutger. Vanuit het verre Smeermaas terug naar Maastricht. Bedankt voor alle geweldige, bizarre avonturen en mooie gesprekken. Dat er in de toekomst nog vele mogen volgen.

Alie en Will, het is zover! Bedankt voor jullie aanhoudende interesse in mij en in mijn onderzoek. Ik vond het super fijn om het met jullie te hebben mogen delen. Ik hoop dat alles goed blijft gaan met jullie en dat jullie nog vele geweldige reisjes in het verschiet hebben. Hebe, bedankt dat je mij jarenlang hebt gesteund in al mijn beslissingen.

Dat brengt mij bij mijn fantastische familie. Welke er altijd voor me zijn en dit alles eigenlijk voor mij mogelijk hebben gemaakt. Pap en mam, ontzettend bedankt voor alles! Al zeurde ik de oren van jullie kop, jullie wisten er altijd wel weer een positieve draai aan te geven. Dat gaf me altijd weer positieve energie om door te gaan. Oma, bedankt dat je me altijd vanaf de zijlijn hebt aangemoedigd! En dat geldt natuurlijk ook voor tante Sjaan! Super bedankt!

Ilona en Robbert, een betere zus en schoonbroer kan ik me niet wensen. Ook jullie bedankt voor alle steun, interesse en gezelligheid! Ik hoop dat de bouw van julie droomhuis spoedig verloopt zodat ik daar snel een drankje kan komen doen! Iloon, heel erg bedankt voor alle tijd en moeite die je in het ontwerpen van de kaft hebt gestoken. Ik ben er erg blij mee!

Dan natuurlijk mijn schoonfamilie: Dieter, Henny, Elke en natuurlijk tante Jo. Bedankt voor jullie steun en interesse. Ik ben erg blij dat jullie me zo enthousiast opgenomen hebben in jullie team! Bedankt voor alles!

Tante Annette en Ome Marcel, Amanda en Emar, bedankt voor alle interesse! Ik vind het erg fijn dat jullie aanwezig gaan zijn op de grote dag!

Dat geldt natuurlijk ook voor jou Rob! Al zien we elkaar niet vaak, de intentie is altijd goed. Ik ben er zeker van dat we binnenkort echt weer een drankje gaan doen in Amsterdam, Maastricht of waar dan ook. Bedankt!

Verder natuurlijk nog iedereen die ik ben vergeten, maar wel op wat voor manier dan ook heeft bijgedragen aan het tot stand komen van dit proefschrift. Dan heb ik het natuurlijk ook over de sfeer op de afdeling, de uitjes, gegeven adviezen, praktische hulp en ga zo maar door. Bedankt iedereen!

Lieve Britt, deze laatste paragraaf is natuurlijk voor jou. Ineens was je daar. Wat ben ik blij dat ik jou heb leren kennen. Mijn grootste motivator en steun in de rug als het nodig was. We hebben samen een fantastische tijd in New York gehad, waar dit boekje ook grotendeels is ontstaan. Fijn dat we nu samen met de hele beestenbende bij elkaar wonen. Ik kijk erg uit naar onze toekomst samen en ben ontzettend blij dat wij elkaar gevonden hebben!

Curriculum Vitae

Nick Petrus van Goethem was born on January 16th 1984 in ‘s-Hertogenbosch, The Netherlands. He finished high school in 2001 at the ds Pierson College in ‘s-Hertogenbosch. Subsequently, he started a Bachelor in Industrial Engineering and Management at Avans Hogeschool in ‘s-Hertogenbosch, which he completed in 2006. In the same year, he started a Bachelor in Psychology at Maastricht University, and successfully completed the Biological Psychology track in 2009. After finishing this Bachelor, he was admitted to the Master track Neuropsychology that same year. For his Master thesis he did an internship in the Mood & Cognition group of Dr. Prickaerts at the Faculty of Health, Medicine and Life Sciences (FHML). His internship entailed validating an automated tracking program for the object recognition task, and validating putative memory enhancing drugs in rats in this same task. In 2010, he obtained his Master’s degree cum laude. Right after his internship he started as a full-time research assistant in the group of Dr. Prickaerts. During this period, he tested different compounds for the pharmaceutical industry, and was able to do experiments which led to publications later on. His main area of focus was the role alpha7 nicotinic receptors play in different memory processes. After one year, in 2011, he was granted with a Kootstra Talent Fellowship for a talented future PhD student. This enabled him to do start his PhD-project at FHML in 2012 under supervision of Dr. Prickaerts and Prof. Dr. Steinbusch. During his Kootstra Fellowship year, and subsequently his PhD trajectory, he continued his research into alpha7 nicotinic receptors and the role different selective ligands play on memory formation and behavior. In 2014 he went to New York City for an internship at Intra-Cellular Therapies, Inc. There, he set up behavioral experiments for the validation of different putative cognition enhancing drugs. Furthermore, he explored different possibilities to investigate re- and desensitization processes in alpha7 nicotinic receptors *in vitro*. For his stay in New York, he received a grant from Alzheimer Nederland. The results of the research performed during his internship, Kootstra Talent Fellowship and Phd-project are described in this dissertation.

Nick Petrus van Goethem werd op 16 januari 1984 geboren in ‘s-Hertogenbosch. Hij voltooide in 2001 zijn middelbare school aan het ds Pierson College in ‘s-Hertogenbosch. Direct hierna startte hij met de opleiding Technische Bedrijfskunde aan Avans Hogeschool in ’s-Hertogenbosch, welke hij afronde in 2006. In dat zelfde jaar, begon hij aan een Bachelor Psychologie aan de Universiteit Maastricht. In 2009 rondde hij de Bachelor track Biologische Psychologie succesvol af. Na de afronding van de Bachelor, werd hij toegelaten tot de master track Neuropsychologie in dat zelfde jaar. Voor zijn Master thesis heeft hij zijn stage gelopen bij de Mood & Cognition groep van Dr. Prickaerts aan de Faculty of Health, Medicine and Life Sciences (FHML). Zijn stage-onderzoek behelsde het valideren van een geautomatiseerd scoringsprogramma voor de object herkenningstaak, en het testen van vermeende geheugenverbeterende stoffen in ratten in dezelfde taak. In 2010 voltooide hij zijn Master opleiding cum laude. Direct na zijn stageperiode startte hij als full-time onderzoeksassistent binnen de groep van Dr. Prickaerts. Gedurende deze periode testte hij preklinisch verschillende stoffen voor de farmaceutische industrie. Verder was hij in de mogelijkheid verschillende onderzoeken uit te voeren welke later tot publicaties hebben geleid. Binnen zijn onderzoek richtte hij zich voornamelijk op de rol die alpha7 nicotine receptoren spelen in verschillende geheugenprocessen. Na één jaar, in 2011, werd hem een Kootstra Talent Fellowship voor een taletvolle aspirant promovendus toegekend. Dit maakte het voor hem mogelijk om een start te maken met zijn PhD-project. In 2012 startte hij met zijn daadwerkelijke PhD-project binnen FHML onder supervisie van Dr. Prickaerts en Prof. Dr. Steinbusch. Gedurende zijn Kootstra Fellowship jaar en opvolgende PhD-traject, is hij doorgegaan met zijn onderzoek naar alpha7 nicotine receptoren en de rol die verschillende selectieve liganden voor deze receptor spelen in geheugen formatie en gedrag. In 2014 is hij naar New York City gegaan om daar een stage te lopen bij Intra-Cellular Therapies, Inc. Aldaar heeft hij gedragsexperimenten opgezet om vermeende cognitie-verbeterende stoffen te kunnen testen in ratten. Daarnaast heeft hij verschillende mogelijkheden onderzocht om re- en desensitisatieprocessen van alpha7 nicotine receptoren te kunnen onderzoeken *in vitro*. Voor zijn verblijf in New York ontving hij een subsidiebeurs van Alzheimer Nederland. De resultaten van zijn onderzoek gedurende zijn Master stage, Kootstra Talent Fellowship en PhD-project, staan beschreven in dit proefschrift.

List of publications

International peer-reviewed journals

van Goethem, N.P., Prickaerts, J., Welty, D., Flood, D.G., & Koenig, G. (2015). Continuous infusion of the α7 nicotinic acetylcholine receptor agonist EVP-6124 produces no signs of tolerance at memory enhancing doses in rats: a pharmacokinetic and behavioral study. *Behavioural Pharmacology,* 26(4), 403-406

van Goethem, N.P., Schreiber, R., Newman-Tancredi, A., Varney, M., & Prickaerts, J. (2015). Divergent effects of the "biased", 5-HT_{1A} receptor agonists F15599 and F13714 in a novel object pattern separation task. *British Journal of Pharmacology,* 172, 2532-2543

Akkerman, S., Blokland, A., **van Goethem, N.P.**, Creemers, P.A., Steinbusch, H.W.M., & Prickaerts, J. (2015). PDE5 inhibition improves acquisition processes after learning via a central mechanism. *Neuropharmacology, In Press*

Van Hagen, B.T.J., **van Goethem, N.P.**, Lagatta, D.C., & Prickaerts, J. (2015). The object pattern separation (OPS) task; a behavioral paradigm derived from the object recognition task. *Behavioural Brain Research,* 285, 44-52

Karamihalev, S., Prickaerts, J., **van Goethem, N.P.** (2014). Donepezil and the alpha-7 agonist PHA 568487, but not risperidone, ameliorate spatial memory deficits in a subchronic MK-801 mouse model of cognitive impairment in schizophrenia. *Behavioural Brain Research*, 272(1), 248-251

van Goethem, N.P., Prickaerts, J., Chesworth, R., Shapiro, G., Boess, F.G., Methfessel, C., Reneerkens, O.A.H., Flood, D.G., Hilt, D., Gawryl, M., Bertrand, S., Bertrand, D., & König, G. (2012). EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacology,* 62(2), 1099-1110

van Goethem, N.P., Rutten, K., van der Staay, F.J., Jans, L.A.W., Akkerman, S., Steinbusch, H.W.M., Blokland, A., van't Klooster, J., & Prickaerts, J. (2012). Object recognition testing: rodent species, strains, housing conditions, and estrous cycle. *Behavioural Brain Research*, 232(2), 323-334

Zeef, D.H., **van Goethem, N.P.**, Vlamings, R., Jahanshahi, A., Hescham, S.A., Prickaerts, J., & Temel, Y. (2012). Memory deficits in the transgenic rat model of Huntington's disease. *Behavioural Brain Research,* 227(1), 194-198

Akkerman, S., Blokland, A., Reneerkens, O.H.A., **van Goethem, N.P.**, Bollen, E., Gijselaers, J., Lieben, C., Steinbusch, H.W.M., & Prickaerts, J. (2012). Methodological considerations on discrimination and exploration measures in object recognition. *Behavioural Brain Research*, 232(2), 335-347

Submitted/Under review

van Goethem, N.P., Fedele, E., Puzzo, D., Rebosio, C., Gulisano, W.,Palmeri, A., Wennogle, L.P. Peng, Y., Steinbusch, H.W.M., Prickaerts, J. (2015). Blocking α7 nicotinic acetylcholine receptors improves specifically memory acquisition. S*ubmitted to Neuropsychopharmacology*

Commentaries

Blokland, A., **van Goethem, N.P.**, Heckman, P., Akkerman, S., Schreiber, R., Prickaerts, J. (2014). Translational issues with the development of cognition enhancing drugs. *Frontiers in Neurology, section Neuropharmacology* 5:1-2

Invited book chapters

van Goethem, N.P., Lardenoije, R., Kompotis, K., Rutten, B., Prickaerts, J., Steinbusch, H.W.M. (2014). "In vivo models for drug discovery", Chapter: "Cognitive Disorders: Impairment, Aging & Dementia". Editors José Miguel Vela, Rafael Maldonado and Michel Hamon. Wiley-VCH Verlag, Weinheim, Germany. 47-364

van Goethem, N.P., Berkers, R., Rutten, K., Blokland, A., & Prickaerts, J. (2014). "Episodic Memory: Formation, Clinical Disorders and Role of Aging", Chapter: "The medial temporal lobe: Toward a unifying neuropsychobiological framework of recognition and recall." Nova Science Publishers, Inc., New York, USA. 85-133

“I am a brain, Watson. The rest of me is a mere appendix.”

Arthur Conan Doyle, (1859-1930)